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STACHYBOTRYS: A MYCOTOXIN-PRODUCING FUNGUS OF INCREASING TOXICOLOGIC IMPORTANCE

Introduction:

Stachybotrys has been recently implicated as a possible agent to a variety of health effects especially among occupants of water-damaged buildings. This review selects published studies and reports regarding Stachybotrys mycotoxins related to human toxicology and summarizes their results and conclusions.

Epidemiology of Stachybotrys:

Human diseases associated with toxins produced by fungi have been described for centuries. Examples are poisoning from food contaminated with *the* fungus Claviceps purpurea and the aflatoxins produced by certain strains of Aspergillus flavus in peanuts and other crops.[1] Due to the potency of aflatoxins, data available on various A flavus strains have revealed that only about 30% of the strains actually produce aflatoxins.[2]

Since the 19th century, there have been reports in the former Soviet Republics of fatal epidemics among farm animals, mainly horses, *after* ingesting moldy hay. The syndrome termed alimentary toxic aleukia (ATA) was used to describe the effects *on* animals and people of presumed mycotoxin poisoning due to ingesting grains contaminated with a mold of Fusarium sp. Further laboratory studies and reports showed that the clinical picture of animals exposed to mycotoxins produced by Stachybotrys sp. was strikingly similar to that of ATA.[3]

Mycology and Toxicology of Stachybotrys:

Mycologically, members of the genus Stachybotrys belong to the family of Dematiaceae. They are cellulolytic saprophytic fungi with worldwide distribution.[4-6] They have been found in soil and substrates rich in cellulose such as hay. *straw.* cereal grains, plant debris, wood pulp, paper, and cotton. This fungus does not compete well with commonly encountered fungi such as Aspergillus and Penicillium. Stachybotrys produces spores *or* conidia which when wet are *sticky* and not easily airborne, and can grow in a wide range of temperatures. The minimal critical levels for growth of fungus are 15% moisture content in the substrates with a relative humidity above 90%. The nomenclature of Stachybotrys has been confusing. For example. Stachybotrys altemans has been changed to S. atra. while S. chartarum has been used interchangeably with S. atra. The identification of a specific *species of* Stachybotrys has not been well defined.[8] The fungus is known to produce several mycotoxins including satratoxins, trichothecenes, and stachybocins.[7-9] However. not all Stachybotrys species produce trichothecenes and under certain conditions. *some* fungi lose the ability to produce toxins.[10] Mycotoxins produced by Stachybotrys are soluble in water and organic solvents.[11]

Stachybotrys Mycotoxicosis:

Drobotko first described stachybotryotoxicosis as a new disease of hones and humans exposed to S. altemans.[12] After several humans exposed their skin to this mold, a rash or dermatitis occurred. Several human volunteers during the same study also complained of epistaxis and "catarrhal angina" of the pharynx. A small number of humans developed leukopenia, but never below 2000 cells/mm3. However, the author concluded in this study that spores of the mold are practically nontoxic.

A comprehensive review of stachybotryotoxicosis was done by Forgacs.[8] Although a detailed account was given regarding the historical. pathological, clinical. toxicological, and microbiological aspects of this fungus, little information was provided on human toxicology. This review implied that most reports on human toxicity from this fungus came from Russian investigators and lacked specific toxicological and epidemiological analyses.[8]

In 1980, Kozak[13] published a paper regarding methods available for "home mold survey." He used a Scotch tape imprint method and found Stachybotrys fungus on *the* carpet of the home of a 4 1/2-year-old asthmatic male who tested positive with fungal extract. The authors did not find viable Stachybotrys by Andersen air sampling method. This report provided no evidence that a causal relationship exists between Stachybotrys and asthma. More importantly, this report did not imply an association of Stachybotrys and mycotoxicosis.

In 1986, Croft[14] published a case report suggesting airborne trichothecene was responsible for illness in a family with subjective mmplaints of "recurring cold and flu symptoms, sore throats, diarrhea, headache. fatigue. dermatitis. intermittent focal alopecia, and general malaise." NO objective clinical and laboratory abnormalities were detected by several physicians despite repeated medical evaluations. The authors found evidence of S. atra from a contaminated dud and in the ceiling. No air sampling was performed and the authors concluded that the symptomatology experienced by the family was due to airborne trichothecenes isolated from *the* fungus. This article has since been cited in later reports as a study of mycotoxicosis secondary to Stachybotrys.[15-17]

Sorenson et al.[15] performed an experiment which demonstrated *the* presence of trichothecenes in aerosolized conidia of S. atra. However, the authors did not investigate animal or human exposures nor establish a relationship between airborne S. atra and pathology.

Several anecdotal events of Stachybotrys exposure were reviewed by Schiefer.["] Calling for more research, *the* author pointed out that a 1987 Canadian Health and Welfare Working Group on Fungi and Indoor Air found that the evidence for a causal relationship between indoor fungal exposure and human disease was weak.

Johanning[17] and his co-workers presented selected data on a group who worked in a mold-contaminated building. Symptomatology was collected using a cross-sectional survey questionnaire. Symptoms. mmplete blood count, T and B cell flow cytometry, mitogen response assay. immunoglobulin panel, angiotensin converting enzyme, lie r enzyme. hepatitis C antibody, Stachybotrys-specific IgG, and IgE radioallergosorbent test were analyzed. The authors found that 7 of 43 respondents had a positive IgE test to other fungi frwn prior exposure. Four had a positive reaction to Stachybotrys IgE antibody. However, the authors did not identify if specific clinical diagnoses were made on the affected workers or whether any diagnosis correlated with a dose response exposure to mycotoxins or allergen produced by S. atra found by air sampling.

In another conference proceeding. Morey[18] reported *that* Stachybotrys was found by air sampling in a "control building' where occupants had no complaints and no visible mold was present. It was presumed that this "control building" had a history of renovation several years prior to *the* study resulting in residual Stachybotrys spores present in the occupied spaces.

A recent article relating Stachybotrys mycotoxin to *adverse* health *effects* was published in 1996.[19] Health complaints of workers from an affected building were identified by a 187-item questionnaire. A test battery including complete blood count, serum chemistries, flow cytometry, lymphocyte function tests. and natural killer cell activity was used to compare symptomatic patients with a control group. Satratoxins from the affected building were identified by high-pressure liquid chromatography. The authors concluded that prolonged and intense exposure to toxigenic S. chartarum and other atypical fungi was associated with self-reported respiratory symptoms (nasal irritation, burning, stuffiness and congestion, sore and burning throat, recurrent shortness of breath, cough, chest tightness, and wheezing) and CNS symptoms (severe headache. concentration problems, irritability. dizziness and light-headedness, sleeping problems, and extreme mental fatigue). They also found a few abnormal parameters pertaining to the cellular and humoral immune *system,* suggesting a possible immune competency dysfunction. However, the paper did not define "prolonged and intense exposure." It did not indicate the potential bias of using a self-reported questionnaire in obtaining symptomatology of workers from the affected building. There was no specific exclusion of other diseases or

conditions causing these symptoms. The nonspecific nature of the immunology studies (flow cytometry, lymphocyte proliferation assays, and natural killer cell activity) was not emphasized And finally, no in vitro or in vivo assays were performed to demonstrate if satratoxins were in fad biologically active at the test site.

A case control study to investigate the risk factors for a cluster of acute pulmonary hemorrhage in infants in the Cleveland area was reported in MMWR.[20] The report indicated that 9 of 10 case infants and 16 of 30 controls resided in households with smokers. The odds ratio (OR) was 7.9. All 10 case infants and 7 controls resided in homes where major water damage had occurred during the previous 6 months and the OR was 16.7. These findings prompted inspection and sampling For and microscopic identification cl fungi.

The quantity of fungi including toxigenic S. atra, according to the report, was higher in the homes of case infants than in those of the control. The calculated OR, however. was only 1.6. The report did not imply a cause-effect relationship between mycotoxin and pulmonary hemorrhage. It further stated that causes for pulmonary bleeding and pulmonary hemosiderosis may include cardiac lesions associated with left atrial pressure, trauma. pneumonia, and perhaps suffocation. Table 1 summarizes published studies related to humans exposed to Stachybotrys.

Reference	Setting			
Drobotko[12]	Human Volunteers	Case Report	Dermatitis, epistaxis	A new disease
Kozac[13]	Asthmatic Child	Case Report	Patient tested (+) for fungal extract to Stachybotrys	Possible allergy
Croft[14]	Family Members	Case Report	"Flu" symptoms S. atra found in evidence of ceiling & duct-specific disease	No objective
Johanning[17]	43 Workers from Mold-Contaminated Building	Cross-sectional	4 had IgE to Stachybotrys, 7 had IgE to other fungi	No specific clinical diagnosis. no control population
Morey[18]	Air-Conditioned Building	Industrial Hygiene Survey	Multiple fungi	"Control" building had identified Stachybotrys
Johanning[19]	53 Workers from Contaminated Office	Cross-sectional	Subjective respiratory and CNS symptoms	No specific clinical diagnosis. no control population
CDC/NIOSH[20]	10 infants with acute pulmonary hemorrage & hemosiderosis	*Case* Control	OR: smoking household 7.9, Water-damaged house 16.9, Presence of S. atra 1.6. Clinical implications	Association of mold growth in water-damaged homes & acute pulmonary hemorrage

There are four ways that a fungus can cause human disease: allergy. mycosis, irritation, and mycotoxicosis. Table 2 outlines a comparison of selected fungi and their potential health effects.

Table 2 - Comparison Chart of Health Effects Due to Fungal Exposure

Fungus	Allergy	Mycosis	Irritation	Mycotoxicosis
Stachybotrys	(+)	([+ or -])	(+)	(+)
Aspergillus	(+)	(+)	(+)	(+)
Penicillium	(+)	(+)	(+)	(+)
Fusarium	(+)	([+ or -])	(+)	(+)
Claviceps	(+)	([+ or -])	(+)	(+)
Candida	(+)	(+)	(+)	(-)
Coccidioides	([+ or -])	(+)	([+ or -])	(-)
Alternaria	(+)	([+ or -])	(+)	(-)

(+) reported, ([= or -]) possible, (-) not reported.

Recent advances in airborne allergic fungi have been reviewed by Burge[21] and Smith.[22] Mycosis is the most common form of fungal disease. It refers to the actual growth of a fungus on a human host implying an infection caused by the fungus. Clinical characterization and management of specific mycosis are well discussed in standard infectious disease textbooks.[23] Irritation can be caused by the mechanical effects of spores and mycelial debris and the volatile organic compounds[24] produced by the fungus.

Mycotoxicosis is an intoxication due to ingestion or inhalation of preformed toxins produced by the mold. It is not necessary for the fungus to be present in the host for illness to occur. Mycotoxins (toxic metabolites) are usually produced after maximum growth of the fungus is reached. Mycotoxicosis occurs typically after consumption of mycotoxin contaminated food on which the mold had grown. Outbreaks of human mycotoxicosis are rare in developed countries. The risk of intoxication increases with improper crop cultivation. food storage. and preparation. Newberne provided a thorough review of the toxicity and carcinogenicity of mycotoxins.[25] Hayes[26] reviewed the biological effects and the role of mycotoxins in human diseases.

The principle mycotoxins of Stachybotrys, called trichothecenes, consist of more than 40 tetracyclic sesquiterpenes that are derivatives of the 12,13epoxy-delta 9-trichothecene ring.[27,28] Stachybocins produced by Stachybotrys sp. have recently been isolated.[9] These compounds contain spirobenzo-furans and terpene units. They are endothelin receptor antagonists and HN viral protease inhibitors.[9,29,30] Other mycotoxins isolated from Stachybotrys are capable of inhibiting pancreatic cholesterol esterase and acyltransferase.[31,32] Several immunosuppressants have been isolated from S. chartarum.[33,34]

The toxic pathophysiology of trichothecenes is similar to toxalbumins such as ricin derived from castor beans.[35] These compounds are potent inhibitors of protein synthesis, especially active at the 80s ribosome subunit. These toxins block protein synthesis at initiation, elongation. and termination phases. Trichothecenes do not need metabolic activation and thus exert toxic effects rapidly.

Recent animal experiments have demonstrated that mycotoxins from S. atra can produce intra-alveolar. bronchidar. and interstitial inflammation with hemorrhagic exudates.[36,37]

Symptoms and signs of trichothecene intoxication are similar to radiation syndrome, affecting rapidly dividing cells in bone marrow and the gastrointestinal tract producing nausea, vomiting, diarrhea, abdominal pain, fever, leukopenia, bleeding. and sepsis. ATA presumably caused by trichothecenes poisoning has been described in four stages[38] outlined in Table 3.

Table 3 - Clinical Stages of ATA

Stage (*)	Onset/Duration	Clinical Picture
1	3-9 days	Nausea, vomiting. mucous membrane and skin irritation, abdominal pain, headache, generalized weakness
2	10-14 days	Patient feels better but bone marrow damage is evident by leukopenia, anemia, and thrombocytopenia
3	3-4 weeks	Coagulopathy, pancytopenia, infection and sepsis
4	>4 weeks	Recovery is marked by increase in WBC count

(*) There is no permanent damage incurred in patients who survive stage 4.

Diagnosis and Treatment:

There are no specific diagnostic symptoms. signs. or laboratory tests for Stachybotrys mycotoxicosis. Austwick[7] suggested that two criteria on which diagnosis must be based are the elimination of other diseases and the presence of an effective level of the mycotoxin that has entered the body. Stabilization, gut decontamination, and supportive treatment for suspected poisoned patients are recommended but are untested. Activated charcoal has been used to treat acute T-2 poisoning.[39]

Discussion:

Stachybotryotoxicosis was first described in Soviet literature when epidemics occurred in which horses died of a mysterious disease characterized by pancytopenia and mucosal ulcerations. Suspected human mycotoxicosis occurred in Russia and other countries as a result of eating moldy and presumed mycotoxin contaminated food.[12,40] In Cambodia, chemical warfare included the use of a trichothecene called T-2[38] also known as "yellow rain." that produced a syndrome similar to ATA: painful skin lesions, dyspnea, lightheadedness, and rapid onset of massive bleeding and death.

Interestingly, after reviewing the literature, the World Health Organization[41] stated that current knowledge is not sufficient to establish a causal relationship between any of the isolated trichothecenes and the outbreak of ATA The report also stated that there have been no reports on the exposure of man to trichothecenes as of 1979.

Research and studies done on the toxicology of mycotoxins have been traditionally related to improving food safety as with ergotism and aflatoxins.[11,42] Most studies done on the toxicology of Stachybotrys mycotoxins have used animal models with an oral route of intoxication. Unlike the literature on ergot and aflatoxin intoxications that includes properly conducted human epidemiology on chronic exposures, clinical case series of acute poisoning. and experimental animal models. human studies on Stachybotrys mycotoxins are limited to case reports, a few poorly controlled cross-sectional studies. and one case control study.[20]

Recently. airborne mycotoxins associated with water-damaged buildings have attracted the attention of health care professionals and the public The controversy over airborne Stachybotrys mycotoxins originated from the extrapolation of data based on case series without specific medical diagnoses. Some reports imply a causal relationship between the presence of Stachybotrys and poisoning. However, to demonstrate a causal relationship between an environmental toxin and its effects, several well-designed epidemiologic studies with sufficient statistical power are necessary.

Future research on Stachybotrys should include the following areas: careful quantitation of the concentrations of mycotoxin in water-damaged vs. "healthy" buildings: documentation of human exposures to known quantities of mycotoxins resulting in clinical syndrome compatible with animal models animals studies to provide evidence for pathogenesis and pathophysiology; and

short-term and long-term health outcomes (not just biologic markers) of individuals who have been exposed to known Stachybotrys mycotoxins.

Summary:

Stachybotrys is capable of producing a variety of mycotoxins that have been shown to *cause* animal and human morbidity and mortality, especially *after* ingestion. Airborne mycotoxins from Stachybotrys in water-damaged buildings *may* produce significant health effects such as pulmonary inflammation, hemorrhage, and immunosuppression.

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Cluster Analysis Applied to Building-Related Illness

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Identifying remediable causes of occupant symptoms in building-related illness is frequently difficult. This is particularly true when the building-wide prevalence of symptoms is comparable to that reported in non-problem buildings. This analysis applied an epidemiological approach to an assessment of a problem building, allowing investigators to visually identify an area of apparent increased symptom density. A cluster analysis approach permitted biostatistical confirmation of the visual cluster. Building-related symptom reporting was statistically significantly associated with a prior physician diagnosis of dust and/or mold allergy. The likely etiology of building occupant symptoms was identified within the region implicated by the cluster analysis. This approach may be useful to focus building evaluations on both the likely physical source and general characteristics of suspect etiologic agents.

Building-related illness continues to challenge both the business and scientific communities. The recommended approach to an investigation of a potential problem building includes an assessment of indoor environmental quality (IEQ) and an assessment of building occupant symptoms. The IEQ assessment[1] targets potential &able problems with environmental temperature, illumination, humidity, and indoor air contaminants. Mold. dust mites, off-gassing from building materials and furnishings, and a long list of materials and processes common to occupied buildings, and entrainment of outdoor contaminants into occupied buildings from nearby industrial operations and parking garages are among the more common pollutants. when no source of pollution can be identified from the IEQ investigation, the designation of "sick building syndrome" is applied. Administration of a standardized questionnaire such as that developed and used by the National Institute for Occupational Safety and Health (NIOSH)[1] attempts to characterize the nature and frequency of symptoms experienced by building occupants.

Sietz categorized the primary factors leading to building-related illness and found that 5% of 529 indoor air quality evaluations conducted by NIOSH from 1971 through 1988 were related to microbial contamination? Fifty-three percent were associated with inadequate ventilation. 15% with contamination sources from inside the building, 10% with contamination sources from outside the building, 4% with contamination from building fabric, and 13% of unknown cause.[2] A variety of clini-

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cal syndromes. including hypersensitivity pneumonitis and humidifier fever, have been associated with a host of fungal, bacterial. and protozoan species dispersed through building heating. ventilation, and air-conditioning (HVAC) systems.' Bioaerosols have also been implicated as a cause of building-related symptoms through a toxic, rather than allergic. mechanism potentially related to effects of endotoxins, mycotoxins, or other microbial products.' From an industrial hygiene perspective.' standing water or wet surfaces identified on walk-through surveys should trigger consideration of environmental sampling for microorganisms and testing building occupants for allergy.

We recently completed an investigation of a medium-sized government office building where building-related symptom frequencies were, if anything, lower than those reported previously in four non-problem buildings[6] or in problem buildings investigated by NIOSH.[1] Concern about potential exacerbation of underlying allergic conditions in index cases led investigators to *administer* a modified NIOSH Indoor Air Quality and Work Environment Symptom Survey. Analysis of questionnaire results allowed the authors to confirm the impression of an association between symptom reporting and self-reported prior allergy to mold and/or dust. A cluster of symptomatic employees was visually identified in one area of the building when locations of asymptomatic and symptomatic building occupants meeting case definitions for building-related symptoms w m identified by different colored pushpins on floor plans of the building. We decided to test the visual cluster utilizing cluster analysis methodology. We believe this study is the first to apply cluster analysis to the evaluation of a problem building. The authors discuss conditions that would be necessary to apply these methods to other building investigations.

Methods

Safety personnel working at a medium-sized government office building became aware of symptomatic complaints of 14 individuals. Symptoms were primarily upper respiratory tract and mucus membrane irritant in nature, and some of the building occupants had specific concerns about allergic responses, such as symptomatic worsening Of preexisting allergic rhinitis or asthma associated with working in the building. These symptoms had been reported as occurring for several years but with increasing frequency over the prior three to four months.

The facility was a single-story structure with a basement. Both the first floor and the basement contained office and laboratory spaces as well as conference rooms. The facility was 42 years old. There had been numerous renovations to the space over the years, including installation of acoustical tiles in a suspended ceiling and conversion of a former bowling alley to office space. There had been a history of water intrusion in the basement of the facility, 10 years previously, due to storm sewer backup. The window wells had additionally been a source of repeated witer intrusion into the basement after heavy rains.

The ventilation for the building was provided .by more than of *50* separate air-handling units. All air-handling units were of a constant volume type, and most were equipped with outside air-economizer controls. Outside air percentages had been determined by temperature measurements to be in the range of 15 to 20%.

To survey building occupants. the NIOSH Indoor Air Quality and Work Environment Symptoms Survey' was modified to address the possibility of a primary problem with building-related allergic response. The three additional questions queried respondents about worsening of sinus, hay fever. or asthma symptoms at work and the need for increased doctor visits or increased medication requirements for these conditions in the previous four months. The questionnaire and a cover letter explaining the nature of the project and assuring building occupants of its confidentiality was sent to the offices of employees as listed in a directory. Several reminders were sent via E-mail. and a second mailing was accomplished to the non-respondents who were identified as employed and currently stationed at the facility.

The modified NIOSH questionnaire was coded and computer data entry screens were created. Double data entry with verification was performed. Decision rules were created with regard to appropriate handling of missing and ambiguous data

Symptoms were deemed to be significant if respondents indicated that they occurred at a frequency of one or more days per week in the last four weeks. Symptoms were further deemed to he building-related if respondents indicated rhat symptoms improved when they were away from work; for example, during holidays or over weekends. These are the same criteria used in prior studies.[1,6]

Questionnaire responses of *293* occupants *(92%* of 320 available employees) were analyzed. Office location was verified by section secretaries for *269* of *293* questionnaire respondents *(92%).* Study participants were once again assured that their questionnaire responses would be mated in a confidential manner. Four case definitions were formulated a priori based on the number of building-related symptoms reported; for example, two or more symptoms. three or more symptoms. four, or more symptoms, and a "sick building–like syndrome." The latter designation required one or more central nervous system (CNS) symptoms and one or more mucus membrane and respiratory irritant symptoms. Irritant symptoms for purposes of this definition included dry. itchy, or irritated eyes, wheezing, sore or dry throat. chest tightness. stuffy or

TABLE 1
Prevalence of Selected Work-Related Symptoms: Comparison of index Building with NIOSH[1] and Washington State Databases[a]

Symptoms	NIOSH Base (%) n = 2436	Washington State Base (%) n = 646	Study Building (%) n = 293	NIOSH Index*	Washington State Index*
Upper respiratory tract/mucous membrane					
Dry. itching. or irritated eyes	30	30	18.	.6	.6
Sore or dry throat	18	14-20	7	.4	.4-.5
Stuffy or runny nose, or sinus congestion	21	21	6	.3	.3
Cough	9	5	2	.2	.4
Lower respiratory tract					
Wheezing	4	2	3	.8	15
Shortness of breath	5	3	1	.2	.3
Chest tightness	6	4	2	.3	.5
Nervous system					
Headache	25	24	9	.4	.4
Unusual tiredness, fatigue, or drowsiness	25	25	to	.4	.4
Difficulty remembering things or concentrating	9	11	5	.6	.5
Dizziness a light-headedness	8	7	1	.1	.1
Skin					
Dry or itchy skin	9	6	5	.6	.8

* Study building prevalence/reference base prevalence.

TABLE 2
Frequency of Building-Related Symptoms Among Those With and Without Dust and/or Mold Allergy (%)

Symptoms	Dust and/or Mold Allergy n = 78	No Dust and/or Y d d Allergy n = 201	Test Statistic; P Value
Dry, itching, or irritated eyes	20 (26%)	31 (5%)	6.91;0.01*
Wheezing (1 missing)	4 (5%)	4 (2%)	0.22†
Headache	11 (14%)	12 (6%)	4.70;0.03*
Sore or dry throat	11 (14%)	8 (4%)	8.96;0.003*
Unusual tiredness, fatigue or drowsiness	12 (15%)	16 (8%)	2.93;0.09*
Cheat tightness	3 (4%)	2 (1%)	0.11†
Sore or runny nose, or sinus congestion	10 (13%)	8 (4%)	0.001†
Cough	4 (5%)	1 (1%)	0.01†
Tired or strained eyes	18 (23)	27 (13%)	3.73;0.05*
Tension, irritability or nervousness	12 (15%)	17 (8%)	1.73;0.19*
Pain or stiffness in back, shoulders or neck	10 (13%)	17 (8%)	0.99;0.32*
Sneezing	8 (10%)	9 (4%)	0.05†
Difficulty remembering things or concentrating	5 (6%)	9 (4%)	0.36†
Dimness or light-headedness	2 (3%)	1 (1%)	0.18†
Feeling depressed	2 (3%)	6 (3%)	1.00†
Shortness of breath	3 (4%)	0	0.02†
Nausea or upset stomach	1 (1%)	2 (1%)	1.00†
Dry or itchy skin	6 (8%)	7 (3%)	2.03;0.15*

* Likelihood ratio Chi-square test, 1 d.f.
† Fisher's exact tart. two-Tail.

not appear to be any building-wide problems with indoor environmental quality.

Table 2 compares the prevalence of building-related symptoms among thox questionnaire respondents who addressed the questions dealing with prior dust and/or mold allergies. Seventy-eight of 279 respondents to these questions indicated that they had a prior physician diagnosed dust and/or mold allergy, while 201 indicated that they bad no such prior diagnoses. This comparison indicated a statistically significant increased reporting of building-dated symptoms in those individuals with prior history of dust and/or mold allergy. In addition, the specific symptom reported were indicative of the symptoms one might anticipate related to allergies, including dry. itchy, or irritated eyes; sore or runny nose, or sinus congestion;

TABLE 3
Prevalence of Symptomatic Questionnaire Respondents In Basement Cluster vs Other Basement and Other Building Locations (n = 269)

Case Definitions	Cluster (n = 45)	Other Basement (n = 97)	Other Building (n = 224)
Sick building–like syndrome	24%	10%	9%
Two or mom building-related symptoms	49%	23%	24%
Three or more building-related symptoms	36%	16%	13%
Four or more building-related symptoms	27%	7%	9%

cough; sneezing; and shortness of breath.

The association between basement office location and symptom reporting was explored for those respondents with two, three, or four or more building-related symptoms. The significance of basement office location was also determined for worsening sinus, hay fever, or asthma symptoms at work and for increased doctor visits and/or increased medication requirements for these conditions in the previous four months. Of these, borderline significance ($0.05 < P < 0.10$) was obtained using 1 degree of freedom Chi-square with continuity correction for two or more building-related symptoms and for worsening sinus, asthma, or hay fever symptoms at work. The relationship between three or more building-related symptoms and basement office location was significant at $P < 0.025$.

Of the 269 questionnaire respondents with verifiable office locations at the time of questionnaire completion, 142 had basement offices and 127 had first floor offices. The area of apparent visual clustering included 42 offices occupied by 45 questionnaire respondents with verified office locations, Table 3 compares the prevalence of the four different case definitions among those included in the apparent cluster as compared with the background prevalence both as represented by other basement locations, apart from the cluster. and when compared with the entire building, apart from the cluster. Observation of these data suggested that the cluster cases could explain the statistically significant increase in cases identified in the basement.

Table 4 shows the results of the two methods for cluster analysis applied to the four case definitions for each floor of the study building. Cluster analysis based on the number of adjacencies was statistically significant only for four or more symptoms in the basement. Statistical significance ($P < 0.05$) was achieved for Method No. 2, for two or more and four or more symptoms in the basement. The coordinates for the geographic center for all four case definitions fell near the apparent center of the cluster identified visually and the location of an office where standing water with visible microbial overgrowth beneath the floor was subsequently discovered. This serendipitous discovery lent empirical support to the findings of the questionnaire evaluation showing a higher prevalence of building-related illness in those workers with basement offices cl among individuals with prior physician diagnoses of dust and/or mold allergy.

Discussion

The results of this investigation are noteworthy in several respects. First. by modifying an existing questionnaire instrument to query respondents specifically about building-associated allergy symptoms. the investigators were able to confirm the impression that prior allergy was associated with symptom reporting and that symptom prevalence was greater among occupants of basement offices.

The modified NIOSH Indoor Air Quality and Work Environment Symptoms Survey identified building-related problems occurring in subgroups of the building population when overall prevalence rates for building-related symptoms were lower than the frequencies reported in the literature for both problem and non-problem buildings. Indexing symptom frequencies to those cited in published data bases helps in identifying potentially important elevations. For instance. a 6% prevalence of building-related wheezing in the subject building should receive more attention than a 30% prevalence of building-related complaints of *dry*. itching, or irritated eyes.

The choice of case definitions for the outcome variables was difficult. Other investigators have attempted to group symptoms in physiologically meaningful clusters, eg. upper respiratory tract symptoms or cutaneous symptoms. After initial evaluation based strictly on number of symptoms, additional analyses to test symptom groupings might add additional clues to potential etiologic agents.

This case study is the first published account of cluster analysis techniques applied to building-associated illness investigation. The data required to perform the cluster analysis are the same needed for a traditional pushpin approach the office locations of "cases" and "non-cases." Superimposition of a grid to assign coordinates and computer data entry and programming are additional procedures required of this approach. The additional effort yields a result that is both more satisfying and convincing than the visual impression alone. Structural features and barriers and differences in population density cause difficulty in adjusting the visual impression of numerator data ("cases") clustering for differences in denominator ("non-cases") clustering. In this specific instance, an area comprised of conference moms contained no "cases" because there were no occupants.

TABLE 4
Cluster Analysis of Building-Related Symptoms for Study Building Occupants, Based on 10,000 Repetitions: First Floor Occupants (n = 127) and Basement Occupants (n = 142)

Case Definitions	Respondents With Condition	Observed Adjacencies (Method #1)	P Value for Adjacencies	Observed Mean Distance to Geographic Center Method #2 (x,y coordinates) Mean Distance	P Value for Distance
Two or more symptoms					
First floor (%)	25 (19.7)	13	0.5755	(27.3. 12.3) 16.0	0.7711
Basement (%)	44 (31.0)	55	0.1348	(34.0, 10.4) 13.7	0.0143
Three or more symptoms					
First floor (%)	14 (11.0)	3	0.7817	(21.7, 11.5) 14.2	0.3630
Basement (%)	33(23.2)	28	0.3157	(32.2, 10.7) 14.0	0.0708
Four or more symptom					
First floor (%)	12(9.4)	1	0.9592	(21.5. 10.5) 15.2	0.6178
Basement (%)	19 (13.4)	16	0.0279	(35.6, 9.2) 12.9	0.0586
Sick building syndrome					
Fim floor (%)	11(8.7)	2	0.7144	(21.9, 13.3) 11.8	0.0807
Basement (%)	21 (14.8)	10	0.5101	(33.6, 10.6) 13.6	0.1039

Another area of unusually small offices appeared to have more cases visually, but this proved to be related to a high population density.

The two approaches utilized in this analysis are based on different measuns of spatial clustering. Method No. 1 is based on the concept of adjacency and allows the investigator to make potentially meaningful decisions about the affect of structural barriers. Such a method would likely yield superior results if walls, corridors, or HVAC system service areas were important factors in pollutant exposure. It is technically more difficult and less reproducible because different investigators may arrive at differing assumptions.

Mcthod No. 2 ignores structural elements and uses distance from the geographic center as its sole measure of central tendency. It has the advantage of being less biased and the disadvantage of lacking sensitivity to geographic barriers. Method No. 2 is more easily generalized to other building investigations requiring only that investigators have questionnaire symptoms data linked to office location and a blueprint of the physical plant. Both methods would likely be less helpful in outbreaks that involve more than one floor or more than one wing, such as a contaminant likely distributed through the ventilation system. On the other hand, evidence of lack of clustering might direct attention to a generalized phenomenon or to multifactorial causation, with several etiologies interacting to cause building-related symptoms from multiple sources of varying location throughout the building. Other etiologies. including symptoms spread through functional interaction of the workforce, eg, infectious disease, or word-of-mouth spread, eg, mass psychological illness, could also be operative and could be discovered with an approach based solely on office location. Thus. whether a specific etiologic source is discovered or not, this appmach *can* localize an area of concern. The walk-through evaluation in search of etiologies and remediation efforts addressing potential toxicologic and psychosocial stressors can be focused.

The authors emphasize that the epidemiologic appmach was critical to the results in this particular case study. Symptom prevalence was not unusually elevated in the building as a whole when compared with problem or non-problem building databases.',' Wen it not for the pushpin epidemiologic methods utilized, a clinically significant and remediable cause for true illness would have escaped recognition. The putative causal nature of the association between the finding of standing water with microbial overgrowth would be strengthened had remediation efforts been completed. Follow-up questionnaire evaluations of building occupants documenting resolution of symptoms would be highly supportive. As of the time of this publication, remediation plans are *still* being finalized.

Acknowledgment

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High-Voltage Gender Neutrality

Duke Energy Corporation recently sent its shareholders an updated version of its prospectus' stock purchase and dividend reinvestment plan. It's a suitably dull document, but one thing caught our eye. In a warning set off in italics on the inside front cover, Duke advises: "All references to the masculine gender in the Prospectus are for ease of reference only, and shall be deemed to refer to both the masculine and feminine gender."

How politically correct can you get? Duke's supervisor of shareholder services knew nothing of this ridiculous exercise in gender neutrality and suggested we ask the chief financial officer, who figured that the firm's general counsel might have an answer. Robert Lewis, Duke's in-house attorney, confesses, "It was probably written by a lawyer. Maybe the very one you're talking to."

—From Transparent Eyeball. *Forbes,* September 8, 1997, p 40.

Occup Environ Med 1998;55:579–5

Correlation between the prevalence of certain fungi and sick building syndrome

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Abstract

Objective—To examine the role of fungi in the production of sick building syndrome.

Methods—A 11 month study in the United States of 48 schools (in which there had been concerns about health and indoor air quality (IAQ)). Building indoor air and surface samples, as well as outdoor air samples were taken at all sites to look for the presence of fungi or their viable propagules.

Results—Five fungal genera were consistently found in the outdoor air and comprised over 95% of the outdoor fungi. These genera were Clodosporium (81.5%), *Penicillium* (5.2%), *Chrysosporium* (4.9%), *Alternaria* (2.8%), and *Aspergillus* (1.1%). At 20 schools, there were significantly more colony forming units per cubic metre (CFU/m³) ($p<0.0001$) of propagules of *Penicillium* species in the air samples from complaint areas when compared with the outdoor air samples and the indoor air samples from non-cornplaint areas. At five schools, there were more, although not significant ($p=0.10$), *Penicillium* propagules in the air samples from complaint areas when compared with the outdoor air samples and the indoor air samples from non-complaint areas. In 11 schools, the indoor air (complaint areas) fungal ratios were similar to that in the outdoor air. In these 11 schools *Stachybotrys* otro was isolated from swab samples of visible growth under wetted carpets, on wetted walls, or behind vinyl wall coverings. In the remaining 11 schools, the fungal ratios and CFU/m³ of air were not significantly different in different areas. Many of the schools took remedial action that resulted in an indoor air fund profile that was similar to that outdoors.

Conclusions—Propagules of *Penicillium* and *Stachybotrys* species may be associated with sick building syndrome.

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Keywords: fungi; sick building syndrome; indoor air quality; spores

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Reports about buildings with air relarcd problems have appeared increasingly ofren after the early 1970s, although this problem has certainly been with humans for centuries.[1,2] Sick building syndrome (SBS), a commonly used term for symptoms resulting from problems with indoor air quality (IAQ), was first recognised as an important problem affecting people in certain buildings in 1982. The first official study of SBS that examined more than one structure was published in 1984.[1] Sick building syndrome has been difficult to define and no single cause of this malady has been identified.[1]

Complaints common to SBS include allergic rhinitis, difficulty in breathing, headaches, Ru-like symptoms, and watering of the eyes.[1] Numerous studies have been carried out in an atrernpt to elucidate the cause of SBS.[1,3] Early studies showed that many of the reported causes of SBS were undesirably high levels of known respiratory irritants such as nitrogen and sulphur dioxides, hydrocarbons, and particulates," known or suspected carcinogens such as asbestos, radon, formaldehyde, and tobacco smoke,[1] or chemicals being released by new building rnarerials.

Alrhough fungal spores are universal atmospheric components both indoors and outdoors and are now generally recognised as important causes of respiratory allergies,[1,4] there are few studies showing which fungi and spores are associated with IAQ problems." This study was made possible due to our association with an JAQ company. The uniqueness of this study was that the sites were made available because the school officials contacted the IAQ company. This allowed us access to all the samples, data, questionnaires, and occupant generated complaints from schools that were experiencing IAQ problems.

Although no one cause for the symptoms induced by IAQ problems is likely to exist, the presence of fungi in sick buildings is becoming consistently associated with this problem.[15-17] Fungal contamination in indoor environments has been shown to produce allergies in occupants of these buildings."[18] Even though the IAQ company's investigations were broadly based and in depth, the presence of fungi was the primary focus of chis study, in which we present evidence for the role of *Penicillium* species and *Stachybotrys* species in buildings with IAQ problems.

Materials and methods

SURVEY PROCEDURES

The 22 month study examined 48 schools that were experiencing IAQ problems. These were in states along the United States Gulf of Mexico and rhe Atlantic seaboard. The sites were surveyed with the following criteria: collection of building characterisation data based on direct inspection and interviews with building occupants; building characterisation including measurement of temperature and humidity, esamination of heating, ventilation,

and air conditioning (HVAC) systems and physical examination of the building; particulates, CO_2, and chemical measurements; inspection of sites that had been wened or showed mould growth; swab samples and air samples taken by an Andersen air sampler; and administration of a questionnaire to building occupants, or access to occupant generated complaints (letters to school officials) and other complaint surveys conducted by other agencies-such as public health departments.

The questionnaire that was administered by the IAQ company was designed to determine the areas or rooms in which the occupants had complaints about the IAQ. The questionnaire asked for such information as the nature of the complaint, symptom panerns, timing patterns, and observations about building conditions that might explain the symptoms found. The questionnaire's answers and comments, along with the occupant generated complaints and surveys from other agencies, were placed into the following categories: *(a)* type of symptoms; (*b*) when do the symptoms start; (*c*) when do the symptoms go away; (*d*) when are the symptoms worst; (*e*); pre-existing symptoms (allergies, asthma, etc); (*f*) discomfort complaints (noise, temperature, odoun, etc); and (*g*) complaint areas (rooms).

Non-complaint areas were also identified, The minimum criteria for designation as a non-complaint area were (*a*) all occupants, whose primary location was the non-complaint area, had no IAQ complaints, and (b) the HVAC system was separate from any complaint areas.

MICROBIOWGICAL IDENTIFICATION

Air samples were taken with a two stage Bioaerosol Sampler (Model 2000 Andersen Samplers, Atlanta, GA, USA) at a calibrated flow rate of 28.4 l/min for 5 minutes. During sampling, the Andersen samplers were placed about 1 m above floor level. Sabouraud's Dextrose agar (SDA) pH 5.6 was used for air sampling and swab sampling. Plates were incubated at 22°C and 90% relative humidity (RH) for up to 14 days. The isolated fungi were identified with standard identification techniques.[20-23] Colony forming units per cubic metre (CFU/m^3) of air were calculated with the formula:

$$CFU/m^3 = (\text{number of CFU} / ((\text{number of minutes sampled})(1\ ft^3/\text{minute}))) \ (35.3\ ft^3/m^3)$$

The total fungal CFU/m^3 for each air sample was calculated and the ratio for each organism per sample was determined. The results were entered according to the area that was sampled (the outdoor air sample areas, the indoor air samples complaint arcas or the indoor air sample non-complaint areas) and the average CFU/m^3 and ratio, in terms of percentage, for each organism was determined for each area examined.

Using sterile swabs, samples were taken from areas of visible fungal growth, HVAC systems, wetted areas, standing water, dead air spaces, and areas of dust accumulation. The swabs were either placed into sterile plastic bags for transport to the laboratory or streaked undiluted on to agar pH 5.6 plates. At the laboratory, the swab tip was placed into a sterile Nbe containing 10 ml sterile phosphate buffered saline and vigorously vortexed for one minute. Samples (100 µl) were pipetted on to agar plates (pH 5.6) and spread with sterile rods. The plates were incubated at 22°C and 90% relative humidity for up to 14 days. The fungi were identified and the fungal growth on the plates was estimated with the following criteria: 0 CFU, no growth; 1–5 CFU, very light growth; 6–10 CFU, light growth; 11–30 CFU, medium growth, 31–50 CFU, heavy growth; and >50 CFU, very heavy growth.

CARBON DIOXIDE MEASUREMENTS

The CO_2 content of the air, expressed in parts per million (ppm), was monitored with a Ventostat CO_2 Sensor (Model 1070/1071, Telaire Systems, Delspo, Sweden).

CHEMICAL MEASUREMENTS

An independent laboratory was contracted by the IAQ company to perform sampling for various indoor air components such as formaldehyde, nitrogen dioxide, hydrogen sulphide, sulphur dioxide, and carbon monoxide. Formaldehyde samples were collected with a Sensidyne/Gastec (Sensidyne/Gastec, Goleta, CA) collector in association with colorimetric indicator tubes with a lower detection limit of 0.1 ppm. Nitrogen dioxide, hydrogen sulphide, sulphur dioxide, and carbon monoxide samples were collected with a MultiRAE PGM-50 gas sampling device (Rae Systems, Sunnyvale, CA, USA). The collector was calibrated before sampling for each particular component. Outdoor and indoor samples were taken in the complaint and non-complaint areas. All sampling was performed in accordance to the manufacturers suggested use.

MEASUREMENTS OF PARTICULATES, TEMPERATURE, AND RELATIVE HUMIDITY

Airborne particles were counted with an APC-1000 Airborne Particle Counter (Biotest Diagnostics, Denville, NJ, USA). The APC-1000 detects particles relative to four thresholds: >0.3, 0.5, 1.0, and 5.0 µm. The APC-1000 also measures temperature and relative humidity.

REMEDIAL ACTION IN BUILDINGS

Any building materials that showed physical deterioration were removed and replaced. Existing microbial contamination on intact and structurally sound surfaces was cleaned and treated with an approved disinfectant. The remedial measures to the HVAC consisted of the removal of visible surface contaminants and the cleaning of air side surfaces of all internal air handling surfaces including, but not limited to, fans, coils, drain pans, filter racks, motors, dampers, and specific air ducts. Any damaged or delaminated insulation within the airducts being cleaned and sanitised was replaced. All work areas inside the air handlers, air ducts, and equipment rooms were isolated and kept under negative air pressure with high efficiency particulate arrestor filtered negative

Incidences per 100 employees (95% CI) of reported complaints and symptoms regarding indoor air quality (IAQ) at 48 United States schools between 1994 and 1996

Type of symptom	Incidence	95% CI	Type of symptom	Incidence	95% CI	Type of symptom	Incidence	95% CI
Nasal drainage and congestion	19.8	±1.3	Discomfort complaints			When are symptoms the worst?		
Itchy or watering eyes	14.3	±1.1	Odorus	5.2	M.4	High humidity	12.0	to.9
Contact problems	5.6	±1.2	Temperature (hot/cold)	7.2	±0.1	Low humidity	0.0	±0.0
Headaches	12.5	±0.6	Noise	0.8	±0.3	Spring	3.9	±0.8
Sinus	10.3	±0.5	Ventilation	6.1	33.3	Summer	*0.0*	±0.0
Severe	3.4	±0.4				Fall	2.7	r0.6
Increased airway infections	14.3	±1.0	Onset of symptoms			Winter	4.5	±0.5
Cough	b.5	±0.6	Entering the building	3.4	m.9	Start of school	5.7	±2.0
Shortness of breath	5.9	m4	Working in the building	11.0	±1.7	Morning	3.4	to.4
Sneezing	6.8	±1.0	Start of school	11.3	L1.9	Afternoon	1.1	±0.3
Dizziness	2.2	±0.5				Monday	*0.8*	M.3
Fatigue	1.1	±0.3	When do symptoms go away?			Late in week	0.8	to.3
Flu-like symptoms	1.8	±0.6	Never	3.5	±0 8	NO pattern	1.1	f0.3
Nausea	1.8	±3.4	Leave work	2.1	±0.6	Always	2.3	to.6
Allergies	17.0	±1.0	Weekends	4.3	10.9	Before remedy		
Asthma	1.4	m3	Vacations	14.7	±2.5	IAQ complaints or symptoms	31.3	±6.8
Other health conditions	1.2	±0.5	Medications	4.4	S . 2	After remedy		
						IAQ complaints or symptoms	2.5	±1.1

air machines to prevent migration of particulates. The cleaning was conducted in accordance with the National Air Duct Cleaners Association Standard 1992-01.[34] All work was done after hours or at weekends. All personnel involved in the remedial work had the proper safety equipment and training and the Occupational Health and Safety Administration standards were observed. Air and swab samples were retaken within 60 days and after at least 6 months of completion of remedial work.

DATA ANALYSIS

Data were analysed by a computer program (Sigma Stat) with the Mann-Whitney rank sums test (*U* test), the Kruskal-Wallis one way analysis of varience (ANOVA) (*H* test), Spearman's product moment correlation, and Dunn's multiple comparisons and partial correlations."

Results

COMPLAINTS

Of the 48 schools surveyed, 40 were elementary schools (children aged 5–10). At most of these sites the school nurse distributed the IAQ company's questionnaire to only the staff. There were 622 occupants that reported IAQ symptoms or complaints, which represented 28% of the total staff (students were not included). With the exception of nausea, there were no significant differences between the reported complaints and symptoms at the different sites. All of the sites were combined and the average incidence per 100 employees along with the 95% confidence intervals (95% CIs) are displayed in the table. Nasal drainage and congestion (IR 19.3, 95% CI ±1.3) and itchy and watering eyes (IR 14.0, 95% CI ±1.1) were always the most common complaints, although all of the symptoms, (with the exception of nausea) listed in the table were reported at each site. Most of the occupants registering complaints stared that their symptoms were a result of either entering or working in the building and most stated that the symptoms usually went away during weekends and holidays and returned on entering the building. More than half of fhe occupants that had IAQ complaints also complained of increased respiratory infections (such as tonsillitis, bronchitis, and some cases of pneumonia) (IR 14.3, 95% CI ±1.0). Over one third of the occupants that registered complaints claimed that an increase in the relative humidity resulted in an increase in the severity of their symptoms (IR 12.0, 95% CI ±0.9).

At most schools, before the IAQ company's investigations, public health departments had conducted investigations and naires. These investigations consisted primarily of measuring the CO, in the buildings and. increasing the ventilation rates, but with no success in reducing the IAQ complaints. The symptoms from the public health departments' questionnaires correlated with the symptoms obtained from the IAQ company's questionnaire. At all schools, many of the occupants that had IAQ complaints had submitted letters to the school officials, detailing their complaints and symptoms. The complaints and symptoms listed in these letters correlated with the complaints and symptoms obtained from the IAQ company's questionnaire.

After remedial action to any building, questionnaires were made available to the staff. They were asked to say if there were any complaints or symptoms related to the IAQ of the building. There were none or only a few complaints concerning the IAQ from the buildings that underwent remedial action. Complaints after the action were never registered by more than 3% of the staff at any school, The overall incidence was 2.5/100 employees (95% CI ±1.1). This represented a significant reduction ($p<0.001$) in the number of IAQ complaints.

CARBON DIOXIDE CHEMICAL AND PARTICULATE

Although CO_2 concentrations were higher indoors than outdoors, there were no significant correlations between the indoor concentrations and the complaints or symptoms (data not shown). All measured constituents were welt within the normal acceptable range of a school or office and there were no significant correlations between the indoor concentrations and complaints or symptoms (data not shown). No correlations were found between fhc outdoor parriculate measurements, the indoor complaint areas, and the non-complaint areas.

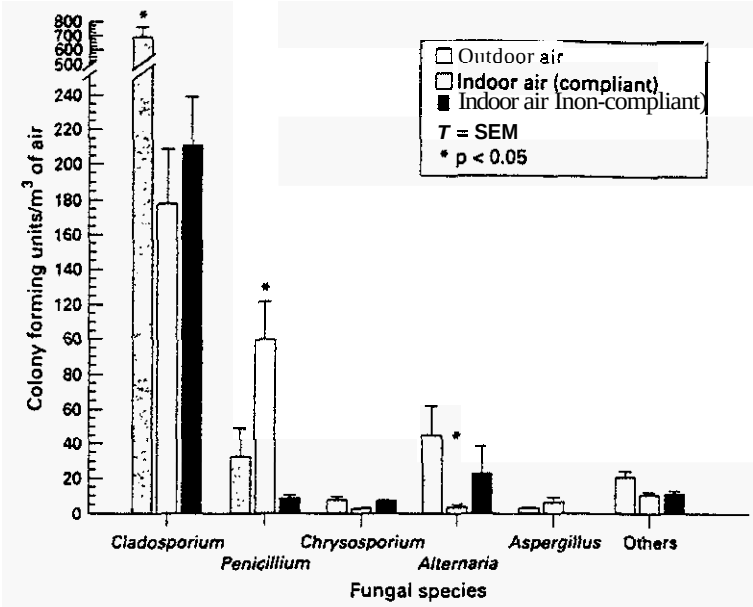

Figure 1 Bar graph of all air samples taken at the 48 schools.

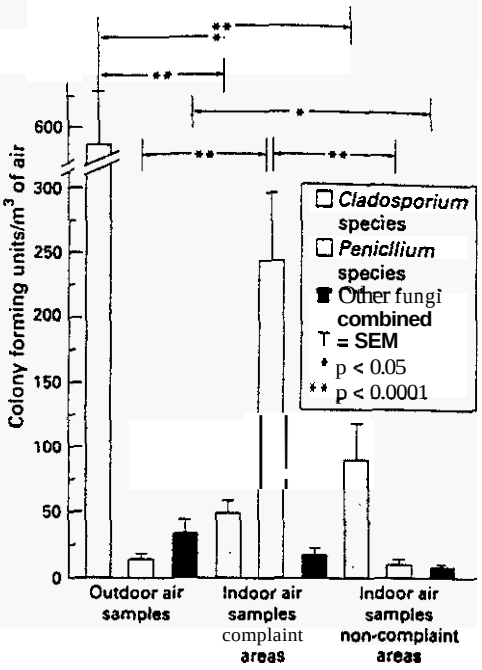

Figure 2 Bar graph of all air samples taken at the 20 schools where Penicillium species were the dominant fungi.

FUNGI IN OUTDOOR AIR

Five fungal genera were consistently found in the outdoor air and comprised over 95% of the outdoor fungi (fig 1). These genera were *Cladosporium* (81.5%), *Penicillium* (5.2%), *Chrysosporium* (4.9%), *Alternaria* (2.8%), and *Aspergillus* (1.1%). Other fungi (*Fusarium, Epicoccum, Botrytis, Bipolaris, Acremonium, Drechslera, Rhizopus, Mucor,* and *Rhodororula)* were present in very low numbers and varied according to location and season. The predominant *Cladosporium* species isolated from the outdoor samples was *Cladosporium cladosporioides.* Several other *Cladosporium* species (such as *Cladosporium herbarum* and *Cladosporium sphaerospermum*) were also isolated, but these isolates were usually found in association with *Cladosporium cladosporioides.* The predominant *Penicillium* species isolated was *Penicillium chrysogenum. Aspergillus niger* was the most commonly isolated *Aspergillus* species from outdoor air samples. With the exception of a few sites along the northern Atlantic coast, most of the buildings were in mild temperate zones, with little or no snowfall, and an average relative humidity range of 30%–60%. The rainfall in the survey areas was not abnormal, with the exception of states surrounding the southern region of the Gulf of Mexico, which were experiencing a drought during the survey period. The outdoor temperature was seasonal, varying from a low near 5°C to a high of 38°C.

INITIAL INDOOR AIR SAMPLES

In most of the schools (fig 1), there were significant reductions in the CFU/m³ of fungi in the indoor air samples from non-complaint areas compared with the outdoor air saniple, but the fungal profiles were similar to outdoor air. In all of the buildings, the CFU/m³ of *Cladosporium* species were significantly ($p<0.05$) lower in the indoor air samples of non-complaint areas than in the outdoor air sample. In the indoor air sample complaint areas, the CFU/m³ of *Cladosporium* species were lower, but not always significantly lower, than the Outdoor air sample. *Penicillium* species and *Aspergillus* species were the only fungi isolated from the indoor air sample complaint areas that had higher CFU/m³ of air when compared with the outdoor air sample and indoor air sample non-complaint arcas. All of the schools bad similar interior temperatures (23°C). Most complaint sites showed very little HVAC maintenance as well as active water leaks.

At 20 schools (fig 2), there were significant increases ($p<0.0001$) in the CFU/m³ of *Penicillium* species in the indoor air samples of complaint areas compared with the outdoor air sample and the indoor air samples of non-complaint areas. The swab samples from these sites had very heavy growth of *Penicillium* species. The mean (SD, range) indoor relative humidity in these complaint areas (IRH-C) was 50% (12%, 23%–67%), in non-complaint areas (IRH-NC) 40% (10%, 30%–48%). The mean (SD, range) outdoor relative humidity (ORH) was 46% (20%, 22%–81%). *Penicillium chrysogenum* was the dominant fungal isolate in 14 of these sites.

In the air samples from complaint areas at five schools (data not shown) there were increases, although not significant ($p=0.10$), in the number of CFU/m³ of *Penicillium* species. In these the IRH-C had a mean (SD, range) of 64% (9%, 54%–70%), the IRH-NC 56% (3%, 54%–58%). The outdoor relative humidity had a mean (SD, range) of 69% (15%, 60%–86%). The swab samples from these sites showed very heavy growth of *Penicillium* species and heavy growth of *Cladosporium* species, which indicated possible fungal growth in the interior and a potential IAQ problem. *Penicillium chrysogenum* was the most common isolate.

At 11 schools (data not shown), *Stachybotrys atra*, which was not isolated from any air samples, was isolated from swabs of the visible growth taken from under wetted carpets, interior painted gypsum board walls, and especially behind vinyl coverings on gypsum board walls that had been wetted in indoor complaint areas. The indoor air samples from these complaint areas were not significantly different from the indoor air samples of the non-complaint areas and had profiles that were similar to the outdoor air samples. The mean (SD, range) of these IRH-C samples was 62% (5%, 58%–66%). The mean (SD, range) outdoor relative humidity was 83% (11%, 65%–90%).

Three *Aspergillus* species (*Aspergillus glaucus*, *Aspergillus versicolor*, and *Aspergillus flavus*) were isolated from interior air samples and swab samples in association with the *Penicillium* species, but *Aspergillus* species (*Aspergillus flavus*) were dominant in complaint areas at only one school. The swab samples from the interior showed heaw to very heaw growth of *Aspergillus* species, along with medium to heavy growth of *Cladosporium* species and *Penicillium* species. The indoor relative humidity at this site was 65% and the outdoor relative humidity was 75%.

In the remaining 11 schools, the fungal ratios and CFU/m³ of air (outdoor and indoor air samples) were not significantly different. The swab samples from the interior of these sites showed heavy to very heavy growth of *Cladosporium* species or *Penicillium* species, which indicated possible fungal growth in the interior. The mean (SD, range) indoor relative humidity was 60% (3%, 56%–64%) and outdoors it was 60% (2%, 57%–62%).

INDOOR AIR SAMPLES AFTER REMEDIAL ACTION

Indoor air samples and swab samples were retaken within 60 days of completion of the remedial action to a building and again at least 6 months after the action. At all sites the fungal ratios (outdoor air samples compared with indoor air samples) were very similar but the fungal CFU/m³ from the indoor air samples were 50%–90% less ($p<0.05$) than the outdoor air samples (data not shown). The indoor relative humidity had a mean (SD) of 44% (5%), with no site exceeding 57%. The outdoor relative humidity ranged from 37%–87%. All swab samples showed very light to light growth of *Cladosporium* species and a mixture of other species.

Discussion

All of the IAQ complaints investigated were generated by the occupants. As the validity of results from questionnaire studies may be altered by biases introduced by the observer or by the respondents," the results must be carefully weighed. Even when observer bias is reduced, the bias introduced by the respondent remains a potential source of systematic error.[26] A major problem, however, is that it is often difficult to control independent variables because of the diversity of the study population, its motility, or a lack of personal exposure. Most of the occupants (90%) in the buildings that we investigated were teachers. Although potential psychological disorders-such as depression or anxiety—were not directly considered, we did, at most schools, find a high job satisfaction and a genuine concern for the welfare of the students, Also, it is difficultto segregate the individual pollutant from copollutants and other confounders. However,. tobacco smoke can be eliminated as a potential confounder as all of the schools prohibited tobacco use on the campuses.

A causal relation is rarely discernible even with strong statistical significance. Thus, at best, associations can be drawn only between the exposure and the effect.

Although fungal contamination in indoor environments has been shown to produce allergies in occupants of these buildings,[16 18 27 28] the role of fungi in IAQ problems has become increasingly controversial. Our studies show that *Penicillium* species and *Stachybotrys* species arc strongly associated with SBS. These data show that the *Penicillium* 'species, especially *Penicillium chrysogenum*, can adapt to an environment in which humans are most comfortable. Our studies also support earlier findings that *Penicillium* species has become an important indoor contaminant.?' This ubiquitous organism's optimal growth occurs between 10° and 25°C. It can grow over a wide range of water availability and has low water activity, although sporulation requires a higher water activity.[30] Whereas it is widely stated that relative humidity >70% is needed for active fungal growth, the water activity of the substrate is actually the critical variable." In the complaint areas where *Penicillium* species were dominant, we found (with the exception of the HVAC system at the fan during cooling) that the range of the indoor relative humidity was from 23%–67%. *Penicillium chrysogenum* is apparently capable of successfully competing with most conidial fungi over almost the entire range of water availability. Its spores are small (1–5 μm) and are capable of entering the lower respiratory tract. It has been shown that bronchial challenges with *Penicillium* species spores induced immediate and delayed type asthma in sensitised subjects."

Stachybotrys species, some of which are capable of producing potent mycotoxins,'? require abnormally high relative humidity or wetted surfaces to grow. This fungus has been associated with illness related to buildings and SBS.[33 34] It is difficult to isolate *Stachybotrys* species from the air and the presence of *Stachybotrys* may have been overlooked due to this phenomenon. Our findings suggest that when the fungal ecology of complaint air samples are similar to the outdoor air and non-complaint air samples, coupled with abnormally high interior relative humidity and symptoms of SBS, the possibility exists that a mycotoxin producing fungus such as *Stachybotrys*, may be hidden and growing in the interior of the building.

Spores of *Cladosporium* species probably occur more abundantly world wide than any

other spore type and are the dominant airborne spores in many areas, especially in temperate climates."[36] Similar to other studies, we found *Cladosporium cladosporioides* growing inside buildings on various building materials."[38] Although our findings showed that *Cladosporium cladosporioides* was not associated with the indoor air sample complaint areas, its presence indicated that the conditions favoured fungal growth that could potentially allow fungal genera such as *Penicillium* or *Aspergillus* to become the dominant organism.

The underlying factor for SBS is the modern, sealed building with its environment controlled by an W A C system. These systems probably contribute to the onset of SBS by allowing build up of pollutants when the capacity of the HVAC system is inadequate or has been compromised. Our finding suggests that the initial stage of interior microbial growth began with water leaks that wetted various building materials. If these wetted materials are not properly mitigated, fungal growth may occur. Eventually, the HVAC system becomes contaminated. Although an understanding of the pollutants or conditions directly responsible for SBS is essential to developing strategies for prevention, a thorough analysis of the W A C system, along with removing or properly repairing wetted areas, were often the key to mitigating the problem in a particular building.

After remedial action, the average air change per hour was 0.5. The air samples taken at 60 days and again at 6 months after remedial action had at least a 50% reduction in the number of CFU/m³ from the indoor air sample than the outdoor air sample. For particles with an aerodynamic size $\geq 2.5\ \mu m$, the current evidence suggests that 1.0 air change per hour results in indoor concentrations of about 30%–80% of that outdoors.[39]

These findings show that remedial action to the buildings removed interior fungal growth. With a significant reduction in complaints about IAQ from occupants, our data suggest that *Penicillium* (*Penicillium chrysogenum*) species and *Stachybotrys* species may be strongly associated with SBS.

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Department of Health Services
Kimberly Belshé, Director

California Morbidity

State of California
Pete Wilson, Governor

Health Effects of Toxin-Producing Indoor Molds in California

Due to excessive rainfall this winter, many Californians are experiencing increased exposure to indoor microorganisms. Several fungal species capable of producing toxic substances have been found in water-damaged California homes and offices. This article provides information about potential health effects from exposure to *Stachybotrys chartarum* (a.k.a. *S. atra*), a toxigenic mold that has received increasing attention recently among indoor air researchers and the public. Within the last 12-18 months, several scientific reports (and media attention) have focused on *Stachybotrys*, a ubiquitous saprophytic fungus that grows on nitrogen-poor, cellulose-rich materials such as hay, straw and building materials (ceiling tiles, wallpaper, paper covering on gypsum wallboard). The statewide prevalence of this fungus in homes or workplaces is unknown, although one report found Stachybotrys in 2-3% of a small survey of southern California homes.[1]

Mechanism of Action

Some strains of *Stachybotrys chartarum* can produce mycotoxins of the trichothecene and spirolactone families. The trichothecene mycotoxins satratoxins G and H am potent protein synthesis inhibitors and cause immunosuppression in laboratory animals. In experimental animal studies, the trichothecenes affect rapidly proliferating tissues such as skin and mucosa, as well as lymphatic and hematopoietic tissues.[2] In laboratory animals, acute exposure to large amounts of trichothecene toxins results in a rapid release of sequestered white blood cells into circulation, while repeated or chronic exposure destroys granulocytic precursor cells in bone marrow leading to white cell depletion. Among the reported cellular effects are: mitogen B/T lymphocyte blastogenesis suppression; decrease of IgM, IgG, IgA; impaired macrophage activity and migration-chemotaxis; broad immunosuppressive effects on the cellular and humoral-mediated immune response leading to secondary infections; and, paradoxically, increased spontaneous antibody producing cells in the spleen.[3]

Toxigenic strains of *Stachybotrys chartarum* may also produce spirolactones (stachybotrylactone) and spirolactams (stachybotrylactam), toxins which produce anticomplement effects.[4] Possible synergistic effects between the trichothecenes and these mycotoxins have not yet been evaluated. Although laboratories can test a sample of *Stachybotrys chartarum* for its ability to produce mycotoxins, *in vitro* results do not necessarily equate with the *in vivo* situation. Therefore, a fungus that produces toxins in the lab may *not* do so in the field, or vice versa. It has been suggested that to assure the safety of any exposed individual, whenever *Stachybotrys chartarum* is identified, it should be considered as a potential mycotoxin-producing organism.[5]

Positive skin reactions to the fungus have been found in some asthmatics living or working in *Stachybotrys*-contaminated rooms, suggesting a hypersensitivity component in addition to the potential for mycotoxicosis. Thus the fungal spores themselves or chemicals carried on the spores may produce either allergenic or toxigenic effects.[6]

Routes of Exposure

Due to its wet, slimy growth characteristics, it is unusual for spores from active *Stachybotrys* colonies to become aerosolized. However, when colonies of this fungus die and become dehydrated, there is increased risk for air dispersion. Portals of possible entry into the body include inhalation and dermal absorption when the fungus is found on walls or in carpets.

Case Reports

Historically, toxicologic effects from this fungus were reported in Europe, where horrcr, sheep and cattle suffered fatal hemorrhagic disorders following Ingestion exposures.[7] Human occupational exposures to contaminated straw or hay resulted in nasal and tracheal bleeding, skin irritation and alterations in white blood cell counts.[7]

The first U.S. case of Stachybotrys-associated health effects from inhalation exposure was reported in a suburban Chicago family.[8] The fungus had contaminated the ventilation system and ceilings of the house. Health effects reported by the family included chronic recurring cold and flu-like symptoms, sore throat, diarrhea, headache, fatigue, dermatitis, intermittent focal alopecia and generalized malaise. Workers who cleaned and removed contaminated material from this house also experienced skin irritation a d respiratory symptoms. After *Stachybotrys* contamination was removed, the house was reoccupied and residents reported no recurrence of clinical symptoms.

Stachybotrys and satratoxin H (one of the trichothecene mycotoxins) were subsequently identified in a water-damaged office building in *New* York City. A small case-control *study* showed workers exposed to tho fungus were at statistically significant higher risk for nonspecified disorders of the lower airways, eyes and skin; fevers and flu-like symptoms, and chronic fatigue.[9,11] NO significant differences in specific *S. charturum* IgE and IgG levels were noted between cases and controls. Although *Stachybotrys charturum* specific IgE (RAST) and IgG (ELISA) tests are available, their sensitivity and specificity have not yet been determined.

A recent report describes identification of 10 likely or possible cases of building-related asthma in a courthouse contaminated with *Stachybotrys* and *Aspergillus* species.[12] Self-reported symptoms among co-workers included fever, headache, rhinitis, coughing, dyspnea and chest tightness. Cheat radiographs were negative and *Stachybotrys*-specific serology was uninformative.

Stachybotrys charturum, along with other fungi and environmental tobacco smoke, was recently postulated to haw an association with pulmonary hemosiderosis in a cluster of Cleveland, Ohio infants.[13,14] While *SC* was found more frequently in tho homes of case infants compared to controls, exposure of case infants *to* mycotoxins in the home could not be determined. Because there is no field test for airborne mycotoxins, it is not currently possible to determine if toxins were actually present in the living space of case infants, and if so, i t what levels. However, since *Stachybotrys charturum* spores containing mycotoxins have been shown to produce pulmonary alveolar and intra-bronchiolar inflammation and hemorrhage in mice,[15,16] mom research into the inhalation effects of these toxins. especially on immature alveoli and pulmonary vascular walls, is critically needed.

Pulmonary hemosiderosis is a condition characterized by recurrent alveolar hemorrhage resulting in clinical signs of cough, wheeze, hemoptysis, tachypnea, low grade fever, and microcytic hypochromic anemia. Chest radiographs typically show patchy infiltrates and sputum specimens, laryngeal swabs or gastric aspirates reveal hemosiderin-laden macrophages. The association of some cases with allergy to cow's milk (Heiner syndrome) and its association with glomerulonephritis In Goodpasture's syndrome suggest an immunologic etiology but immunologic findings in idiopathic cases have been inconsistent. Some familial case reports also suggest a genetic component.

California Department of Health Services staff reviewed statewide hospital discharge data for 1989-1995 (last year for which data are available) and identified 6 total of eight hospitalizations and no deaths during these years for hemosiderosis in infants less than one year of ago. There were no more than 3 cases in any year and no geographic clustering.

On April 6, 1996, the American Academy of Pediatrics (AAP) Committee on Environmental Health released a statement concerning toxic effects of indoor molds and acute idiopathic pulmonary hemorrhage in infants. They recommend that until more information is available on tho etiology of this condition, physicians should try to ensure that infants under 1 year of ape are not exposed to chronically moldy, water-damaged environments.[14]

Sources of Additional Information/Assistance:

Environmental Health Investigations Branch, California Department of Health Services: Sandra McNeel, D.V.M.; Debra Gilliss, M.D., M.P.H.; Richard Kreutzer, M.D. (510) 450-3818

Fact Sheet "Mold in My Home: What Do I Do?" Indoor Air Quality Section California Department of Health Services, 2151 Berkeley Way (EHLB) Berkeley, CA 94704 *www.dhs.ca.gov/org/ps/deodc/ehlb/faqs/* 510-540-2476 (copy enclosed).

Informative website on Stachybotrys maintained by Case Western Reserve University, where Cleveland children with pulmonary hemorrhage were cared for: *http://gcrc.cwru.edu/stachy.*

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AMERICAN ACADEMY OF PEDIATRICS

Committee on Environmental Health

Toxic Effects of Indoor Molds

ABSTRACT. This statement describes molds, their toxic properties, and their potential for causing toxic respiratory problems in infants. Guidelines for pediatricians are given to help reduce exposures to mold in homes of infants This is a rapidly evolving area and more research is ongoing.

ABBREVIATIONS. SIDS, sudden infant death syndrome; CDC, Centers for Disease Control and Prevention.

The growth of molds is pervasive throughout the outdoor environment. Given the proper conditions, molds may also proliferate in the indoor setting. Because Americans spend 75% to 90% of their time indoors,[1] they are exposed to molds that are growing indoors.

Molds readily enter indoor environments by circulating through doorways, windows, heating, ventilation systems, and air conditioning systems. Spores in the air also deposit on people and animals, making clothing, shoes, bags, and pets common carriers of mold into indoor environments. The most common indoor molds are *Cladosporium, Penicillium, Aspergillus,* and *Alternaria.*[2,3]

Molds proliferate in environments that contain excessive moisture, such as from leaks in roofs, walls, plant pok, or pet urine.[4–6] Many building materials are suitable nutrient sources for fungal growth. Cellulose substrates, including paper and paper products, cardboard, ceiling tiles, wood, and wood products, are particularly favorable for the growth of some molds. Other substrates such as dust, paints, wallpaper, insulation materials, drywall, carpet, fabric, and upholstery commonly support mold growth.[3] Molds also may colonize near standing water.[7,8]

Some indoor molds have the potential to produce extremely potent toxins called mycotoxins.[10–12] Mycotoxins are lipidsoluble and are readily absorbed by the intestinal lining, airways, and skin.[13] Species of mycotoxin-producing molds include *Fusarium, Trichoderma,* and *Stachybotrys.* In general, the presence of these molds indicates a long-standing water problem.

DIRECT TOXIC EFFECTS FROM MOLD EXPOSURE

The toxic effects from mold exposure are thought to be associated with exposure to toxins on the surface of the mold spores, not with the growth of the mold in the body. Until recently, there was only one published report in the United States linking airborne exposure to mycotoxins with health problems in humans.[14] This report described upper respiratory tract imitation and rash in a family living in a Chicago home with a heavy growth of *Stachybotrys atra* (also known as *Stachybotrys chatarum*). The investigators documented that this mold was producing trichothecene mycotoxins. The symptoms disappeared when the amount of mold was substantially reduced.

More recently, molds that produce potent toxins have been associated with acute pulmonary hemorrhage among infants in Cleveland, Ohio.[15] In November 1994, physicians and public health officials in Cleveland reported a cluster of eight cases of acute pulmonary hemorrhage and hemosiderosis that had occurred during January 1993 through November 1994 among infants in neighborhoods of eastern metropolitan Cleveland.[16] Two additional cases were identified in December 1994. Pulmonary hemorrhage recurred in five of the discharged infants after they returned to their homes; of these infants, one died from pulmonary hemorrhage.

A case-control study comparing those 10 infants who had acute pulmonary hemorrhage and hemosiderosis with 30 age-matched control infants from the same area in Cleveland[17] revealed that the infants with pulmonary hemorrhage were more likely to have resided in homes with major water damage from chronic plumbing leaks or flooding (95% confidence interval = 2.6 to infinity). The quantity of molds, including the toxigenic fungus *Stuchybotrys atra,* was higher in the homes of infants with pulmonary hemorrhage than in those of controls. Simultaneous exposure to environmental tobacco smoke a p peared to increase the risk of acute pulmonary hemorrhage among these infants.

Stachybotrys atra requires water-saturated cellulose-based materials for growth in buildings. In studies conducted in North America, it has been found in 2% to 3% of home environments sampled.[8,18] Although *Stachybotrys atra* has been associated with gastrointestinal hemorrhaging in animals that had consumed moldy grain,[19] the fungus previously had not been associated with disease in infants. Infants may be particularly susceptible to the effects of these inhaled mycotoxins because their lungs are growing very rapidly. In an *animal* model, intranasal administration of toxic spores of *Stachybotrys atra* to mice resulted in severe interstitial inflammation with hemorrhagic exudates in the alveoli.[20]

The county coroner re-examined all infant deaths

The recommendations in this statement do not indicate an exclusive course of treatment or serve as a standard of medical care. Variations, taking into account individual circumstances, may be appropriate.

in Cleveland during January 1993 through December 1995 to determine whether pulmonary hemosiderin-laden macrophages were present in the lung tissue. Postmortem examinations were reviewed for all 1295 infants who died during that period, including 117 deaths attributed to sudden infant death syndrome (SIDS). Pathologic lung specimens were sectioned, stained with Prussian blue, and screened for the presence of hemosiderin. The presence of hemosiderin-laden mauophages in alveoli indicates alveolar bleeding at least 2 days before death.''

Hemosiderin-laden macrophages were abundantly present in the lung tissue of nine (5%) infants. Of these nine deaths, two resulted from homicide, and one had a recent history of child abuse. The other six deaths that were accompanied by hemosiderin-laden macrophages in the lung thus may have been misclassified as deaths from SIDS. All six infants had lived in the same limited geographic area as the previously described cases of pulmonary hemosiderosis.

The extent of this problem in other areas of the United States is still unknown. Further investigation is needed to establish causation and prevent further health effects if the findings in Cleveland are confirmed in other areas.

CONCLUSION

Very little is currently known about acute idiopathic pulmonary hemorrhage among infants. This is a newly recognized problem and knowledge is expected to be evolving rapidly. In view of the severity of the problem, environmental controls to eliminate water problems and to reduce the growth of indoor molds are wise. Until more is known about the etiology of idiopathic pulmonary hemorrhage, prudence dictates that pediatricians try to ensure that infants under 1 year of age are not exposed to chronically moldy, water-damaged environments.

Coroners and medical examiners should consider using the recently published *Guidelines for Death Scene Investigation of Sudden, Unexplained Infant* Deaths, which includes a question about dampness, visible standing water, or mold growth.

Little is known about the prevalence of toxigenic molds in homes, nor is it clear how extensive measures must be to achieve environments sufficiently free of molds to avoid disease. Bulk mold must be removed, followed by a thorough cleaning with soap and water. Caution must be used, because it is possible that homeowners could actually increase the levels of mold spores in the air by attempting extensive clean-up efforts without guidance from a professional (a certified industrial hygienist or ventilation engineer). These specialists can be found in the yellow pages in the telephone directory under the listing for Industrial Hygiene Consultants. Additional research is needed before the most appropriate recommendations for home clean-up can be determined, Until then, interim guidelines have been formulated.

RECOMMENDATIONS

1. In areas where flooding has occurred, prompt cleaning of walls and other flood-damaged items with water mixed with chlorine bleach, diluted four park water to one part bleach, is necessary to prevent mold growth. Never mix bleach with ammonia. Moldy items should be discarded.
2. Pediahicians should ask about mold and water damage in the home when they treat infants with idiopathic pulmonary hemorrhage. If mold is in the home, pediatricians should encourage parents to try to find and eliminate sources of moisture. Testing the environment for specific molds is usually not necessary. It appears to be important to clean up moldy conditions before the infant is discharged from the hospital to prevent recurrent pulmonary hemorrhage, although this needs further study. Interim clean-up guidelines are available through the Centers for Disease Control and Prevention (CDC), 1600 Clifton Rd, Atlanta, GA 30333.
3. Infants with idiopathic pulmonary hemorrhage must not be exposed to environments in which smoking occurs.
4. Pediatricians should report cases of idiopathic pulmonary hemorrhage and hemosiderosis to state health departments. A reporting form is available through the CDC.
5. Pediatriaans should be aware that there is currently no method to test humans for toxigenic molds such as *Stachybotrys* or mycotoxins.
6. Infants who die suddenly without known cause should have an autopsy done including a Prussian blue stain of lung tissue to look for the presence of hemosiderin.

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Study of Toxin Production by Isolates of *Stachybotrys chartarum* and *Memnoniella echinata* Isolated during a Study of Pulmonary Hemosiderosis in Infants

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A cluster of cases of pulmonary hemosiderosis among infants was reported in Cleveland, Ohio, during 1993 and 1994. These unusual cases appeared only in infants ranging in age from 1 to 8 months and were characterized by pulmonary hemorrhage, which caused the babies to cough up blood. A case-control study identified major home water damage (from plumbing leaks, roof leaks. or flooding) as a risk factor for development of pulmonary hemorrhage in these infants. Because of an interest in the possibility that trichotliecene mycotoxins might be involved in this illness, a number of isolates of *Stachybotrys chartarum* were grown in the laboratory on rice, and extracts were prepared and analyzed both for cytotoxicily and lor specific toxins. Two isolates of *Memnoniella echinata*, a fungus closely related to *S. chartarum*, were also included in these studies. *S. chartarum* isolates collected from the humes were shown to produce a numbrr of highly toxic compounds, and the profiles of toxic compounds from *M. echinata* were similar; the most notable difference was the fact that the principal metabolites produced by *M.* echinara were griseofulvins.

Toxigenic and nontoxigenic strains of *Stachybotrys chartarum* (Ehrenb. ex Link) Hughes (= *Stachybotrys atra* Corda) have been isolated from cellulose-based agricultural materials, including hay and straw, and from confaminated moist building materials (30). *S. chartarum* is the etiologic agent of stachybotryotoxicosis, a severe disease of large domestic animals in eastern Europe. This disease is associated with contaminated straw, is characterized by leukopenia. hemorrhage, and arrhythmic heartbeat, and often lends to death (12). *S. chartarum* is known to produce several mycotoxins (Fig. 1 to 3), including the macrocyclic trichothecenes, which are potent cytotoxins (15), as well as a variety of immunosuppressant agents (2, 16, 24, 25, 34–37). Since the macrocyclic trichothecenes as a group are highly toxic and produce biological effects in experimental animals similar to those observed in stachybotryotoxicosis (15), they are considered the chemical agents responsible for the disease (8). These naturally occurring toxic sesquiterpene metabolites are potent inhibitors of protein synthesis in eukaryotic organisms, and the spores of *S. chartarum* have been shown to contain macrocyclic trichothecenes (39). Recently, there has been increased interest in this organism as a potential cause of adverse health responses in humans in agriculture, homes, and offices (1, 20–22) following the report of Croft et al. (5) that exposure to *S. chartarum* was the apparent cause of an unexplained outbreak of illness over a period of several years in a home located in suburban Chicago, Ill. In the mid-1990s. a cluster of cases of pulmonary hemosiderosis were reported in Cleveland, Ohio (10). These unusual cases occurred only in infants ranging in age from 1 to 8 months and were characterized hy pulmonary hcmorrliage. which caused the babies to cough up blood. A case-control study identified major home water damage (from plumbing leaks, roof leaks. or flooding) as a risk factor for development of pulmonary hemorrhage in these infants (29). Because of an interest in the possibility that trichothecene mycotoxins might be involved in this illness, a number of isolates of S. *chartarum* and *Memnoniella echinata* (a fungus closely related to *S. chartarum*) isolated from homes of infants in the case-control study were grown in the laboratory on rice, and extracts were prepared and analyzed both for cytotoxicity and for specific toxins.

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MATERIALS AND METHODS

Sources of fungi and production of mycotoxins on rice. Cultures of S. *chartarum* and *M. echinata* (Riv.) Galloway were isolated from air and surface samples from homes enrolled in a case-control study of pulmonary hemosiderosis in infants (10, 29). Sixteen representative isolates of *S. chartarum* and two isolates of *M. echinata* were grown on Uncle Ben's Converted Rice (l00 g per 500-ml Erlenmeyer flask) for 4 weeks at 24°C. Two isolates of *S. chartarum* (JS6301 and JS6307) were also grown for 2 weeks at 24°C and then for 2 weeks at 5°C. Subcultures of the following four isolates used in this study have been deposited in the American Type Culture Collection: *M. echinata* JS6308 (= ATCC 200581) and *S. chartarum* JS5802 (= ATCC 201210). JS5817 (= ATCC 2012111. and JS5818 (= ATCC 201212).

Media and chemicals. Media used for maintenance of fungus cultures were obtained from Difco Laboratories (Detroit, Mich.). Minimum essential medium (MEM). dimethyl sulfoxide, and MTT [1-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were obtained from Sigma Chemical Co. (St. Louis. Mo.), and the extraction solvents and solvents used for chromatography were obtained from Fisher Scientific Co. (Pittsburgh, Pa.) and were high-performance liquid chromatography (HPLC) grade.

Preparation or rice culture extracts. All extraction procedures were done in a chemical fume hood. After incubation. the rice was air dried and was stored at

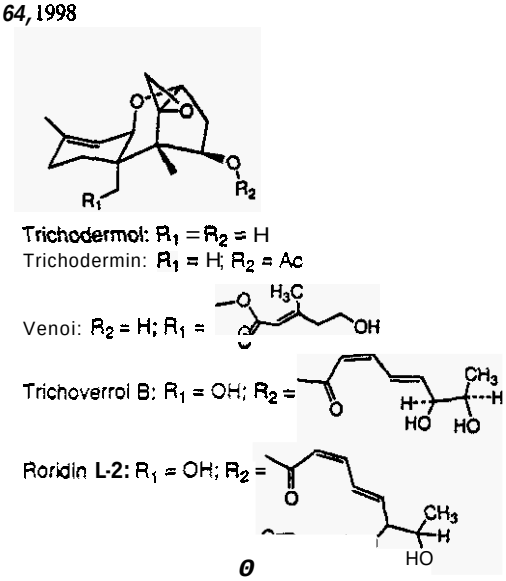

FIG. 1. Chemical structures of trichoverroid trichothecences. Ac, acetyl.

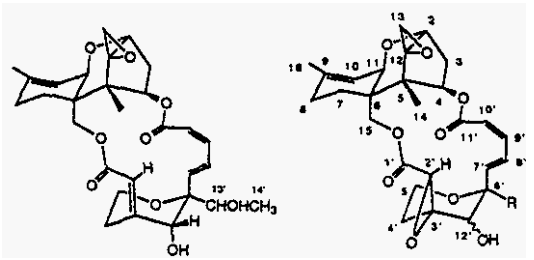

FIG. 2. Structures of satratoxin G, satratoxin H and isosatratoxins F and G.

4°C. The rice was ground with a coffee grinder and covered with methanol (MeOH) overnight. The mixture was filtered, and the solid portion was reextracted twice with MeOH at 40 to 50°C with sonication (Bransonic 321; Branson Sonic Power Co., Danbury, Conn.) for 30 min each time. The MeOH extracts were combined, dried by rotary evaporation. and weighed. Then ca. 100 mg of the crude extract was taken up in ca. 1 ml of $CHCl_3$ and applied to a short column containing silica (about 2.5 g; diameter, 40 to 60 μm) packed in hexane. Three 15-ml fractions were obtained by washing the column with 90% diethyl ether–hexane (fraction F-1), 6% MeOH–CH_2Cl_2 (fraction F-2). and MCOH (fraction F-3). Fractions F-1, F-2, and F-3 represented ca. 20, 10, and 70% of the weight, respectively.

Cytotoxicity testing of culture extracts. Extracts were tested for cytotoxicity by using an inhibition-of-cell-proliferation assay with a continuous cell line of feline fetus lung cells (7). This procedure involved evaluation of mitochondrial activity after treatment with a tetrazolium dye. Extracts were dissolved in MEM containing 10% MeOH. Blank, solvent control wells and serial 1:2 dilutions of samples in MEM were prepared. Cells were added at a concentration of 4×10^3 cells/well. The plates were incubated for 5 days at 37°C in a humidified environment in the presence of 5% CO_2. After incubation, 125 μg of MTT was added to each well, and the plates were incubated for an additional 4 h. The plates were centrifuged, the medium was removed. 100 μl of dimethyl sulfoxide war added. and the plates were shaken for 30 min and read with a plate reader (Dynatech Laboratories Inc., Chantilly, Va.) at 540 nm. The results were recorded as the lowest concentration that resulted in 50% inhibition.

HPLC analyses. Fractions F.1 and F-2 were analyzed by reversed-phase (C_{18}) HPLC with a Gilson model 303 gradient chromatograph. Two systems were used. (i) In the first system, the HPLC instrument war connected to a Knauer variable-wavelength detector (set at 260 nm) and a Shimadzu Chromatopac C-R3A, Supelco 5 μm C_{18} column (4.6 by 250 mm), and chromatography was performed with a 10-min 60 to 75% MeOH gradient in 5% aqueous acetic acid (AcOH) with a flow rate of 1.2 min (solvent system A). (ii) Alternatively, the HPLC instrument was connected to a Shimadzu model SPD-M10A diode array detector and a Beckman Ultrasphere 5 μm C_{18} column (4.6 by 250 mm), and chromatography was performed with a solvent system (Row rate, 0.8 ml/min) consisting of acetonitrile (AcCN) in water by using the following step gradient: 50% AcCN from zero time to 10 min, 75% AcCN from 10 lo 15 min, and 85% AcCN from 15 to 22 min (solvent system B). Levels of trichothecenes were determined With standard curves prepared by injecting known amounts of standards and measuring the areas under the peaks. The following retention timer were observed with solvent system B: trichoverrol B, 4.0 min; roridin L-2, 5.4 min; satratoxin G. 7.3 min: isosatratoxin G, 7.6 min; ratratorin H, 8.7 min; isosatratoxin F, 9.4 min; and roridin E. 16.3 min. The levels of the phenylspirodrimanes were estimated from peak areas obtained with solvent system A. These spirocyclic compounds typically had retention times in the 15- to 20-min range and often produced overlapping perks. Under the same solvent conditions (solvent system A), the trichothecenes had retention times in the 4- to 9-min range.

Water extraction of large-scale culture. *S. chartarum* IS5818 was grown on rice at the ambient temperature for 30 days The culture (1 kg) was coarsely ground and then slurried with water and treated with ultrasound for 30 min. The mixture was filtered by using Whatman no. 1 filter paper, and the rice material was reextracted twice, which resulted in 4.15 liters of a green opaque solution. The liquid was filtered once more and divided into three equal portions, and each portion was then passed through an RP Sep-Pak column (Waters C_{18}, 10 g, 35 ml), in which the trapped compounds were visible as an orange band. The column was eluted with 50 ml of water and then with 100-ml portions of 20% MeOH–water, 70% MeOH–water, and 100% MeOH, and the solvent was removed by rotary evaporation (bath temperature, <35°C). HPLC analysis revealed that the majority of the macrocyclic trichothecenes eluted in the 70% MeOH–water fractions. The 70% MeOH–water fractions were combined and concentrated to dryness. This resulted in 1.5 g of brown gum, which was adsorbed onto polyethyleneimine (PEI) silica (particle size, 40 μm; 1.7 g; J. T. Baker, Phillipsburg, N.J.) and flash chromatographed with a PEI column (30 g; 25 by 180 mm; increasing CH_2Cl_2 in hexane). This resulted in nine fractions which were combined based on a thin-layer chromatography analysis (Fig. 4).

Fraction 1 was applied to a 1-mm Chromatotron plate (Harrison Research Inc., Palo Alto. Calif.) (radial chromatography). and a trace (<1 mg) of roridin E was eluted with 70% ethyl acetate (EtOAc)–hexane. Fraction 2 was purified by 1-mm radial chromatography (EtOAc/hexane/MeOH, 80:20:2), which gave isosatratoxin F (3.3 ms). satratoxin G (14.3 ms), and roridin L-2 (8.3 mg). In an identical manner. fraction 3 gave satratoxin H (8.5 mg). Fraction 4 was applied to a 2-mm chromatotron plate and eluted with 70% EtOAc–hexane, whish gave isosatratoxin G (4.0 mg) and verrol (18) (8.8 mg). Fraction 8 was purified on a 1-mm plate (EtOAc/hexane/MeOH, 85:15:5), which gave trichoverrol B (4.4 mg). Further extraction of the rice culture with methanol and MeOH/$CHCl_3$ (1:1) gave (after filtration and solvent removal) a brown gum (21.4 g). Column chromatography performed with a PEI silica (16) step as a key separatory step yielded. after radial chromatography cleanup. the following major macrocyclic trichothecene constituents: roridin E (23 mg). satratoxin H (16.0 mg), satratoxin G (16.9). and roridin L-2 (5.4 mg).

Isolation of isosatratoxin F. MeOH extraction of a 250-g culture of *S. chartarum* IS5106 that had been grown at room temperature for 30 days gave 7.5 g of crude extract. This material was subjected to medium-pressure liquid chromatography with 150 g of silica gel (Whatman LPS-1). Elution with dichloromethane gave 520 mg in the first fraction, which was subjected to high-speed countercurrent chromatography with a model CCC-1000 instrument (Pharm-Tech Research Corp., Baltimore, Md.) (V_c, 355 ml; Raw rate. 1.8 ml/min; solvent, MeOH/H_2O/CCl_4/hexane/CH_2Cl_2 [6:4:8:1:1]); this resulted in five fractions. Fraction 4 was recrystallized from CH_2Cl_2–hexane, which gave 20 mg of isosatratoxin F (mp. 153 to 155°C; $[\alpha]_D^{25}$ = 46.4° [c = 0.4 acetone]; HUMS [Cl] for $C_{29}H_{34}O_{10}$, calculated 542.2152, found 542.2155: IR {$CHCl_3$} 3478, 1747, 1713. and 1188 cm^{-1}); ^1H nuclear magnetic resonance (NMR) ($CDCl_3$, Bruker AMX at 400 MHz) δ 0.80 (3H, s, H-14), 1.70 (3H, s. H-16). 1.80-2.00 (4H, m, H-7 and H-8). 2.20 (ddd. 1H, J = 4.7, 5.1, 15.5 Hz, H-3β), 2.50 (dd, 1H. J = 8.0, 15.5 Hz, H-3α), 2.80 and 3.12 (AB, 1H each, J = 4.0 Hz, H-13), 3.38 (1H, s, H-2'), 3.54 (1H, d, J = 5.0 Hz, H-11) 3.62 (1H, s, H-12'), 3.83 (1H, d, J = 5.1 Hz, H-2), 4.14

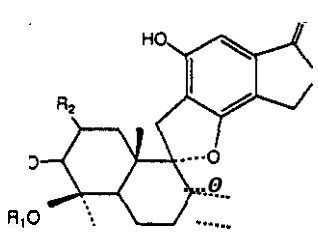

FIG. 3. Structure of phenylspirodrimanes. Ac, acetyl; OAc, acetate.

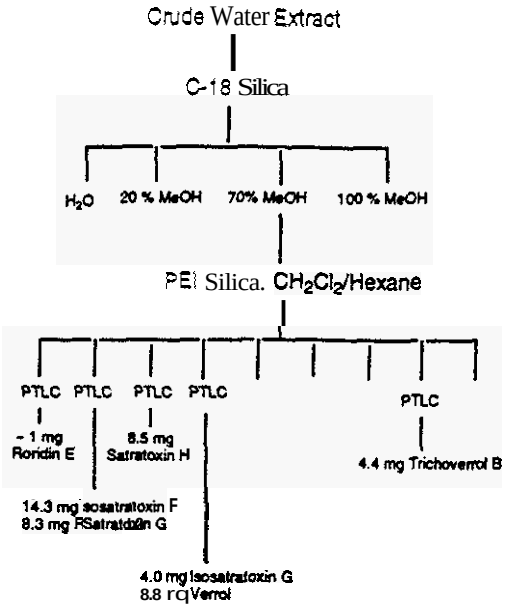

FIG. 4. Flow chart depicting the procedure used for isolation of *S. chartarum* mycotoxins. PTLC, preparative thick-layer chromatography.

(2H, m, H-5'), 4.20 (2H, s, H-15), 5.39 (1H, d, J = 5.0 Hz, H-10), 5.55 (1H, dd, J = 1.6 and 16.4 Hz, H-7'), 5.84 (1H, dd, J = 4.7 and 8.0 Hz, H-4), 5.90 (1H, dd, J = 1.6 and 11.6 Hz, H-10'), 6.54 (1H, ddd, J = 1.6, 5.7, and 11.6 Hz, H-9'), and 6.72 (1H, ddd, J = 1.6, 5.7, and 16.4 Hz, H-8'); ^{13}C NMR ($CDCl_3$, Bruker AMX at 100 MHz) δ 79.1 (C-2), 34.4 (C-3), 74.1 (C-4), 49.4 (C-5), 43.0 (C-6), 20.0 (C-7), 27.4 (C-8), 140.5 (C-9), 118.5 (C-10), 67.7 (C-11), 65.3 (C-12), 47.9 (C-13), 7.9 (C-14), 65.0 (C-15), 23.3 (C-16), 166.0 (C-1'), 58.8 (C-2'), 63.8 (C-3'), 22.5 (C-4'), 61.1 (C-5'), 87.0 (C-6'), 129.8 (C-7'), 130.5 (C-8'), 143.2 (C-9'), 121.1 (C-10'), 166.9 (C-11'), 73.5 (C-12'), 208.6 (C-13'), and 27.4 (C-14').

RESULTS AND DISCUSSION

In general, the levels of cytotoxicity observed (Table 1) correlated with the relative levels of macrocyclic and trichoverroid trichothecenes (Fig. 1 and 2); five of the six cultures that exhibited the highest levels of toxicity (50% inhibitory concentrations, 0.2 to 1.2 μg/g) contained the highest levels of trichothecenes, whereas cultures that exhibited low levels of cytotoxicity contained small amounts or no detectable amounts of trichothecenes. Of the isolates from case and control homes (Table 1), three belonging to each group were among the most toxic and three belonging to each group were essentially nontoxic. Thus, there was no perceived relationship between cytotoxicity and the origin of an isolate (a case home or a control home). Most of the rice culture extracts of *S. chartarum* were found to contain macrocyclic trichothecenes (e.g., satratoxins) and trichoverroid trichothecenes (e.g., roridin L-2 and trichoverrol B) (Table 1). In the HPLC analyses we used a diode array detector which detected the phenylspirodrimanes (Fig. 3) and only those trichothecenes that exhibit UV absorption (e.g., the satratoxins and trichoverroids). It should be noted that the analytical method employed (HPLC with UV detection) did not detect the normally UV-transparent simple trichothecenes, such as trichodermol and verrucarol and their acetates, compounds (Fig. 1) which are known to be produced by *S. chartarum* (17). The phenylspirodrimanes were present in all of the *S. chartarum* and *M. echinata* cultures and were generally present at higher levels than the trichothecenes, but there was no relationship between their levels and cytotoxicity. No attempt was made to determine the levels of the phenylspirodrimanes, but their levels could be estimated from the total peak areas observed in the chromatograms. The phenylspirodrimanes exhibit characteristic UV spectra (λ_{max}, ca. 216, 257, and 300 nm) and are generally well-separated from the trichothecenes in terms of retention time.

Cytotoxicity assays were performed with crude extracts. The crude extracts were passed through a short silica gel column, which resulted in three fractions: fractions F-1 (90% ether–hexane), F-2 (6% MeOH–CH_2Cl_2), and F-3 (MeOH). Moderately polar fraction F-2, which contained the bulk of the macrocyclic and trichoverroid trichothecenes, as well as the phenylspirodrimanes, was subjected to HPLC analyses. Additional cytotoxicity investigations of the fractions demonstrated that the greatest cytotoxicity was found in fraction F-2, although fractions F-1 and F-3 were also appreciably cytotoxic (data not shown). Further studies are under way to determine the origin of the cytotoxicities in these fractions. The results suggest that we are far from characterizing all of the toxins produced by the fungi studied.

Following a series of chromatography procedures (see above), a 250-g rice culture of *S. chartarum* JS5106 gave 20 mg of a compound which appeared to be the previously described compound satratoxin F (9). This amount is significantly more of this compound than we expected based on our analysis of a small culture (Table 1), which indicates that there may be significant variation in toxin production by a given isolate. The NMR spectral data for this compound matched very closely the NMR spectral data for satratoxin F. Most of the carbon chemical shifts for this compound (isosatratoxin F) (Fig. 2) are within 0.2 ppm of the carbon chemical shifts for the corre-

TABLE 1. Chemical and cytotoxicity analysis of Cleveland isolates of *S. chartarum* and *M. echinata*

Strain	Toxicity (μg/ml)	Concn (μg/ml) of: Trichoverrol B	Roridin L-2	Satratoxin G	Isosatratoxin G	Satratoxin H	Isosatratoxin F	Roridin E	All compounds	Concn of phenylspirodrimanes[a]
JS5106[b]	1.2	20	25			10	25		80	+++
JS5119[b]	1.2		20				15		35	++
JS5802[b]	0.3	40	30			10			80	++
JS5108[b]	40								0	+
JS5807	160					20	20		40	++
JS5808	0.2	65	40	15		50			170	+
JS5815	20								0	+
JS5816	>500						5		5	+
JS5806[b]	160								0	+
JS5105[b]	>500								0	+++
JS5111[b]	10		15						15	++++
JS6307[b]	320								0	++
JS6307[b,c]	40								0	+++
JS5817	0.2		40		55	65			160	++*
JS5818	0.2		50	10	5	10	10	25	110	++
JS5819	10					8			8	++
IS6301	80			5		5			10	+++
JS6301[c]	40								0	+++
JS6308[d]	160								0	+
JS6309[d]	160								0	+

[a] +, 10 to 20 ppm; ++, 40 to 90 ppm; +++, 100 to 200 ppm; ++++, 200 to 500 ppm.

[b] Strains obtained from case homes. The other strains were obtained from control homes.

[c] The culture was incubated at 10°C for the final 2 weeks.

[d] *M. echinata* strains. The major products are ca. 5,000 ppm of dechlorogriseofulvin and ca. 5,000 ppm of epidechlorogriseofulvin, as well as trichodermol and trichodermin (total concentration, ca. 200 ppm) (19).

sponding carbons in satratoxin F (9); the only exceptions are the carbon 13' shift (for satratoxin F, C-13' is 217.0 ppm; for isosatratoxin F, C-13' is 208.6 ppm) and the carbon 14' shift (for satratoxin F, C-14' is 29.7 ppm: for isosatratoxin F, C-14' is 27.4 ppm). However, in the proton spectrum, most of the signals for the two compounds are virtually the same; the only exceptions are the H-15 signal (for satratoxin F, H-15 is 3.38 ppm; for isosatratoxin F, H-15 is 4.20 ppm) and the H-12' signal (for satratoxin F, H-12' is 4.24 ppm: for isosatratoxin F, H-12' is 3.62 ppm). Although these data indicate that satratoxin F and isosatratoxin F are diastereomers. they do not allow us to say at which stcreogcnic center(s) (C-12' or C-6') they differ.

Previous investigators reported that some macrocyclic trichothecenes could be extracted from grain artificially contaminated with *S. chartarum* by using swine stomach and intestinal fluid (14, 33). Sorenson et al. (40) reported that aqueous wash preparations of conidia of both *S. chartarum* and *M. echinata* were nearly as toxic as MeOH extracts of the spores themselves. These findings suggest that the somewhat lipophilic trichothecenes can be extracted with water. To examine this further, a 1-kg rice culture of *S. chartarum* JS5818 (not dried) was extracted with water by using sonication. We obtained only about 5% of the normal weight of extract, hut the extract was very rich in trichotheccncs and more than 50 mg of pure trichothecenes was isolated (see above). Based on the total amount of trichothecenes isolated from this culture, this represented about 50% of the trichothecenes produced by *S. chartarum*. The H-13 epoxide hydrogens, which exhibit a characteristic set of AB resonances centered around 3.0 ppm, could be clearly seen in the ^{1}H NMR spectrum of the crude aqueous extract; there was no discernible sign of the trichothecenes in the crude MeOH extract The trichothecenes are exported to the surface of a fungal spore, where they become water soluble, perhaps because they are imbedded in the water-soluble surface polysaccharides. This could be highly relevant to pulmonary hemosiderosis in infants since the highly potent toxins would be readily released into the microenvironment of the developing lung cells in vivo. There were only traces of the lipophilic phenylspirodrimanes in the aqueous extract, which may have been a result of poorer transport of these compounds across the outer fungal cell membrane. There are, in fact, reasons to believe that toxigenic fungi in general export their toxins to the surface, where they can effectively inhibit competition from other microorganisms (6). JS5818 was the only *S. chartarum* isolate found to produce appreciable amounts of roridin E. Interestingly, this isolate also produced both satratoxin G and isosatratoxin G, which proved to be easily separable by chromatography with PEI silica, a chromatographic medium that we have found to be particularly useful for isolation of natural products (16).

S. chartarum and *M. echinata* arc morphologically and physiologically closely related cellulolytic fungi; both of these organisms have worldwide distributions, are often found together. and are commonly found in soil (23). In both the genus *Stachybotrys* and the genus *Memnoniella* conidia are found on clusters of unbranched phialides borne on simple or branched conidiophores. The conidia are dark green to black. The two genera differ primarily in the arrangement of the conidia, which are aggregated in slimy heads in the genus *Stachybotrys* and in long persistent chains in the genus *Memnoniella*. Although these similarities have led some authors to suggest that the species of these genera should be combincd in the same genus, most authors agree that the two genera are distinct (23). The conidia of *S. chartarum* are ellipsoidal and are 7 to 12 by 4 to 6 μm. Although these measurements seem to suggest that the conidia arc too large to enter the respiratory tract, the aerodynamic diameter is ca. 5 μm (39.40). This is consistent with studies which have shown that nonspherical particles, such as fibers, orient themselves in an air stream in the long dimension; i.e., the aereodynamic diameter corresponds to the narrow dimension. *M. echinata* conidia have a smaller aerodynamic diameter (40) and would be expected to have an even grcatcr potential to pcnctratc deep into lungs than the conidia of *S. chartarum*.

The genus *Stachybotrys* has rceivcd considerable attention in the scientific literature, especially in recent years, as a possible health risk in indoor air, but little information is available on the genus *Memnoniella*. Our results indicate that *M. echinata* can have toxicity similar to that of some isolates of *S. chartarum*, although the isolalcs of *M. echinata* which we studied were less toxic than the most toxic isolates of *S. chartarum* studied. We found macrocyclic trichothecenes in cultures of *S. chartarum* but not in cultures of *M. echinata*, demonstrating that the latter species contains some of the toxins of *S. chartarum* but perhaps not all. On the basis of our results, it is not possible to say that *M. echinata* cannot produce macrocyclic trichothecenes since only two isolates were studied (40). *M. echinata* was found in only one sample from the Cleveland homes, and the two isolates, isolates JS6308 and JS6309 (Table 1), appeared to hc indistinguishable. These isolates were moderately cytotoxic and produced the simple trichothecenes trichodermol and trichodermin, as well as substantial amounts of dechlorogriseofulvins (19). The latter metabolites are well-known antifungal compounds produced by several *Penicillium* species but have not been reported to be produced by members of other fungal genera (3), although there is a preliminary report that suggests that *griseofulvins* may be produced by *Aspergillus* species (13). Acareful survey showed that only one of the *S. chartarum* isolates (JS5806) produced any detectable quantities of dechlorogriseofulvins. However, this isolate produced only about 5 ppm of dechlorogriseofulvins. Like other workers (25), we found that our isolates of *M. echinato* also produce phenylspirodrimanes, although at relatively low levels. It should be noted that the macrocyclic trichothecenes are typically considerably more cytotoxic than either the simple trichothecenes, the phenylspirodrimanes, or the griseofulvins. Thus, in terms of their chemical products, both *S. chartarum* and *M. echinata* produce phenylspirodrimanes, but these two organisms differ in that the former produces macrocyclic and trichoverroid trichothecenes and the latter produces griseofulvins. Both produce varying amounts of simple trichothecenes, such as trichodermol and trichodermin. The smaller aerodynamic diameter of the conidia of *M. echinata* and the fact that *M. echinata* produces many of the same toxins suggest that *M. echinata* should also be considered potentially dangerous in indoor air (40). In cases in which both species are present in the same samples, their combincd toxic potential should be considered

It is now appreciated that the principal nonpathogenic biological agents responsible for the health problems associated with damp buildings arc fungi rather than bacteria or viruses (26, 27). Although fungi have been viewed traditionally as allergens (and in unusual circumstances, pathogens) in this context, data have accumulated which show that the adverse health effects resulting from inhalation of fungal spores are due to multiple factors. One factor associated with certain fungi is low-molecular-weight toxins (mycotoxins) produced by the fungi (28). Reports have indicated that airborne spores of toxigenic *S. chartarum*, *Aspergillus versicolor*, and several *Penicillium* species are potentially hazardous. especially when air-handling systems are heavily contaminated (11, 38). Nikulin

and coworkers have provided powerful evidence supporting the notion that mycotoxins in the spores of *S. chartarum* are hazardous. In their work, mice treated intranasally with highly toxic spores of *S. chartarum* died very rapidly compared with mice which received the same dosage of relatively noncytotoxic spores. Both the in vitro cytotoxicities and the in vivo toxicities correlated closely with the levels of satratoxins in the spores (31, 32). Interestingly, studies of inhalation of T-2 toxin (a simple trichothecene with toxicity similar to that of the satratoxins) demonstrated that although this mode of toxin administration was about 1 order of magnitude more effective than intravenous administration, lung tissue of the treated animals remained essentially unchanged *(4)*. This is in stark contrast to what was observed in the lung tissue of mice treated with spores of the highly cytotoxic organism *S. chartarum*; in the latter case the lung tissue had profound lesions (31. 32).

There is extensive literature concerning toxicoses (usually in animals) associated with *S. chartarum* that dates back to the 1930s (12). The reports came mainly from Eastern Europe. although there have been scattered reports of stachybotryotoxicosis in other parts of the world. The fan that no similar cases of stachybotryotoxicosis have been reported in North America may leave the impression that the toxigenic potential of North American isolates of *S. chartarum* differsubstantially from the toxigenic potential of *S.* chananrm strains isolated in Europe. Our data obtained with the Cleveland isolates clearly show that North American isolates of *S. chartarum* have about the Same spectrum of toxigenicity as the isolates described in European studies, although the specific toxins may vary with locality. Eastern European isolates of *S. chartarum* appear to produce commonly satratoxin H as the major macrocyclic trichothecene metabolite, whereas in the Cleveland isolates of *S.* chartarum satratoxin H was the major macrocyclic trichothecene metabolite in only3 of the 16isolates examined. Roridin L-2 was found to be somewhat more common in the Cleveland isolates. Although there was a clear association between the presence of *S. chartarum* and the incidence of pulmonary hemosiderosis in the Cleveland cases, much work has to be done to establish a cause-and-effectrelationship.

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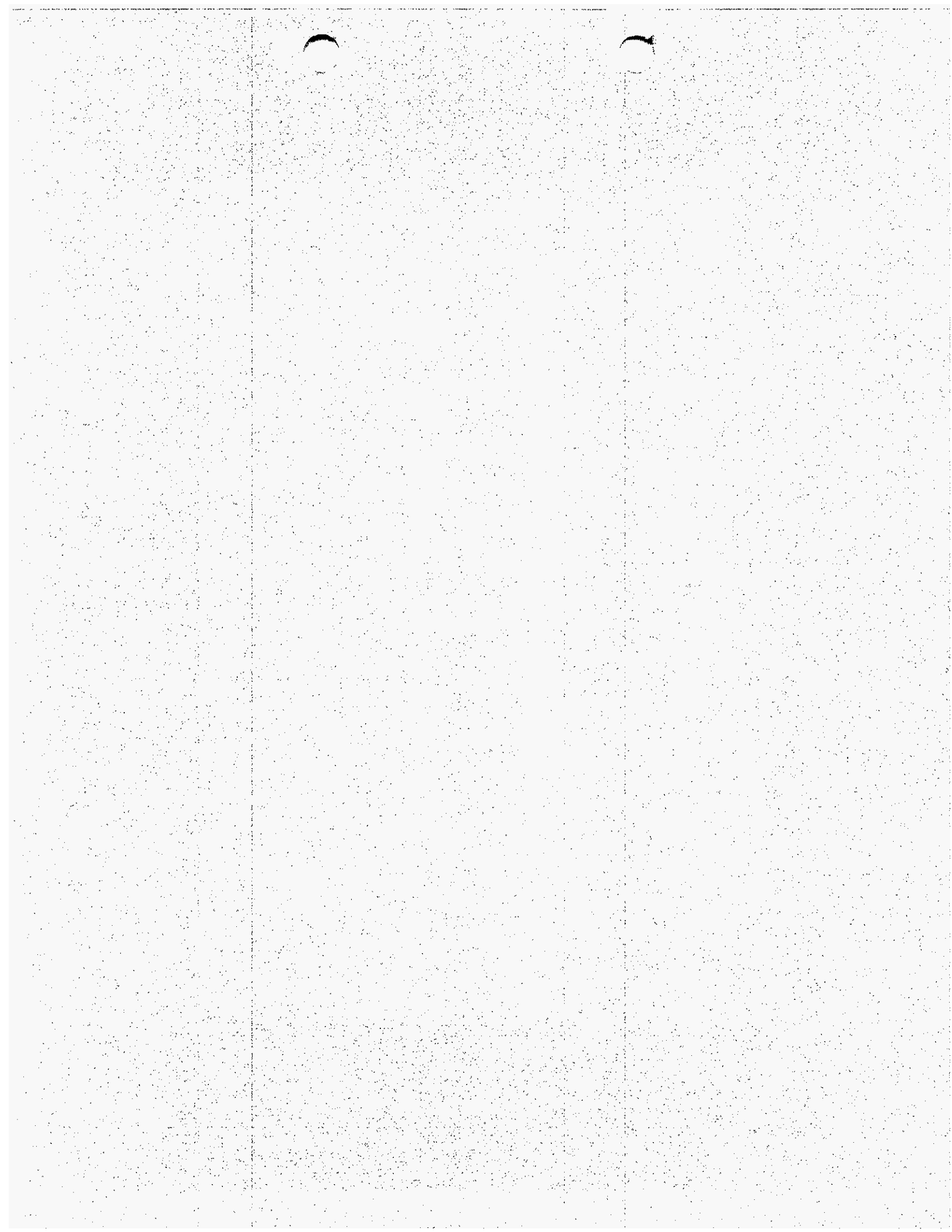

Acute Pulmonary Hemorrhage in Infants Associated With Exposure to *Stachybotrys atra* and Other Fungi

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Background: A geographic cluster of 10 cases of pulmonary hemorrhage and hemosiderosis in infants occurred in Cleveland, Ohio, between January 1993 and December 1994.

Study Design: This community-based case-control study tested the hypothesis that the 10 infants with pulmonary hemorrhage and hemosiderosis were more likely to live in homes where *Stachybotrys atra* was present than were 30 age- and ZIP code–matched control infants. We investigated the infants' home environments using bioaerosol sampling methods, with specific attention to *S atra*. Air and surface samples were collected from the room where the infant was reported to have spent the most time.

Results: Mean colony counts for all fungi averaged 29 227 colony-forming units (CFU)/m³ in homes of patients and 707 CFU/m³ in homes of controls. The mean concentration of *S atra* in the air was 43 CFU/m³ in homes of patients and 4 CFU/m³ in homes of controls. Viable *S atra* was detected in filter cassette samples of the air in the homes of 5 of 9 patients and 4 of 27 controls. The matched odds ratio for a change of 10 units in the mean concentration of *S atra* in the air was 9.83 (95% confidence interval, 1.08-3 × 10⁶). The mean concentration of *S atra* on surfaces was 20 × 10⁶ CFU/g and 0.007 × 10⁶ CFU/g in homes of patients and controls, respectively.

Conclusion: Infants with pulmonary hemorrhage and hemosiderosis were more likely than controls to live in homes with toxigenic *S atra* and other fungi in the indoor air.

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Editor's Note: The epidemiology story is great reading. Rather than 'Eleven Blue Men," we have 10 blue infants.

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PULMONARY hemosiderosis is a rare condition characterized by spontaneous pulmonary hemorrhage, often associated with iron-deficiency anemia.[1] The cause is most often unknown, although previous reports have linked a portion of childhood cases with cardiac or vascular malformations, infectious processes, immune vasculitis, trauma, or known milk protein allergies.[2]

During January 1993 to December 1994, 10 infants with acute pulmonary hemorrhage and hemosiderosis were seen at Rainbow Babies and Childrens Hospital, Cleveland, Ohio.[3] The diagnosis was made by demonstrating alveolar hemosiderin-laden macrophages in biopsy specimens or in bronchoalveolar lavage 3 to 6 weeks after the initial hemorrhage. All of the infants were black, and all but 1 of the infants were male. During the previous 10 years, 3 cases of idiopathic pulmonary hemosiderosis had been diagnosed among infants and children at this hospital.

The purpose of this investigation was to determine the cause of the high incidence of acute pulmonary hemorrhage and hemosiderosis among infants in Cleveland during these 2 years. The affected infants all lived in an area of eastern metropolitan Cleveland within 6 miles of the hospital.

Among the most striking features of this illness were its severity and its tendency to recur after hospital discharge. In 5 infants, acute hemoptysis necessitating readmission to the intensive care unit recurred within 1 day to 6 months of discharge. All infants' hemorrhages were so severe that they required admission to the pediatric intensive care unit. All but one underwent intubation. One infant died. In a previous report from this investigation, infants with pulmonary hemorrhage and hemosiderosis were found to be 16 times more likely than control in-

MATERIALS AND METHODS

All infants younger than 1 yur who had been admitted to the hospital with idiopathic pulmonary hemorrhage and hemosiderosis between January 1993 and December 1994 were included. For each patient. we generated a list of potential controls from all infants born in Cleveland within 2 weeks of the patient and presently living in an area buring 1 of the 6 ZIP codes in which all of the patients lived. The list of potential controls was generated from Cleveland hinh certificates and records of the hospital continuity clinic. Infants' parents were telephoned to invite their panicipation in the study. For uch patient. the first 3 potential controls whore parents agreed to participate were enrolled. Informed consent was obtained from the parents or guardians of all infants.

A pediatrician (R.A.E., E.M., or D.G.D.) visited the homes of all patients and controls to administer a questionnaire comprising more than 200 items that included questions about the infant's health, infant arc practices. and home environment characteristics. The questionnaire included specific questions about the infant's exposure to toxic agents (pesticides, paints. solvents. and gasoline) and structural characteristics such as water damage. A registered sanitarian performed an environmental survey of each home. with special attention to the infants' sleeping and living areas. The pediatrician and the sanitarian were not blinded to the case or control status of the infant.

From December 11 to December 19, 1994, at a separate visit, industrial hygienists unaware of case or control status of the homes performed environmental sampling to ook specifically forthe presence of *S atra* in the air and on surfaces. Residents were not at home during the sampling.

ENVIRONMENTAL SAMPUNG METHODS

Bioaerosol sampling was performed at the homes of patients and wntrols to determine the presence of *S atra* and other fungi. All air and surface samples collected from a ch site for viable microorganisms were refrigerated at approximately 4°C before analysis.

The air samples were collected during 1 to 2 hours from the room where the infant was reported to have spent the most time. Various activities, including vacuuming carpets, pounding on furnace ducts several times, and walking on "pets wcrc performed at each residence in an effort to simulate household activities that could release dusts from ventilation systems and household surfaces. Air samples were collected to test for *S atra* spores and viable fungi.

AIRBORNE CONIDIA

Airborne conidia (spores) were collected using total dust sampling on cellulose ester membrane filters. Samples were collected using a Gilian pump (Gilian Instrument Corporation, Wayne. NJ) at a flow rate of 1.0 L/min for 6 to 8 hours. After sampling, uch filter was positioned on a glass slide. and the entire area of ach filter section was scanned using brightfield microscopy (approximate magnification, ×200) to identify the presence of *S atra* spores. A standard reference slide of *S atra* spores was prepared in the same manner to aid in the identification of spores.

VIABLE AIRBORNE FUNGI

Continuous samples for viable fungi were collected using the CAMNEA filter method.[7] Fungal propagules were collected on polycarbonate filters using a Gilian pump at a flow rate of 2.0 L/min for approximately 1 to 2 hours. By culturing serial dilutions of the filter washings, fungal spores from these samples were enumerated.[7] Diluted filter fluids were plated on the following media: rose bengal streptomycin agar,[8] cellulose agar (Czapek-Dox agar with sucrose and $FeSO_4$ omitted. containing 20 g/m powdered cellulose and 50 mg/L rose bengal and adjusted to pH of 8.0), 2% malt extract agar. and dichloran glycerol agar.[9] The plates were then incubated at 24°C for 10 days. Colonies were classified into the following categories: *Aspergillus, Cladosporium, Penicillium, Stachybotrys*, and other. The other category included all other fungi observed. Concentrations are reported as colony-lormingunits per cubic meter of air sampled (CFU/m³).

SURFACE SAMPLES

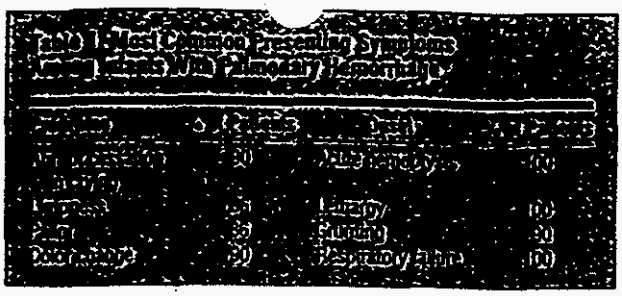
Samples wcrc collected from areas of suspected mold growth in homa of patients and controls by scraping surface materials into sterile centrifuge tubes or plastic bags. Serial 10-fold dilutions were prepared after adding 0.5-g portions of the sample to 49.5 mL of phosphate-buffered saline containing 0.1% polysorbate 80. Aliquots of these dilutions were plated as described, except that 2% malt agar was not used. The plates were incubated at 24°C for 10 days, the colonies were counted. and results wcrc expressed as CFU per gram.

STATISTICAL ANALYSIS

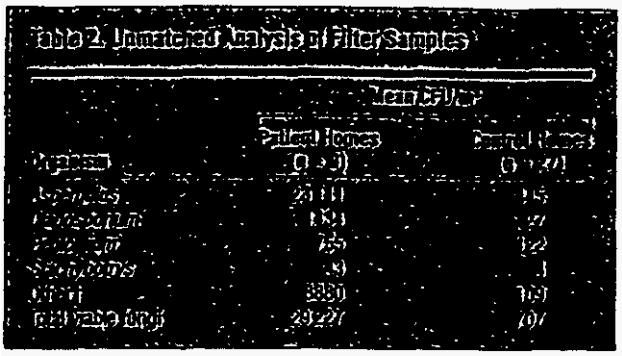
Mean concentrations of fungi in the air and on surfaces were calculated for the homes of patients and controls by dividing the total number of CFU by the number of plates from ach home. LogXact[11] was used to calculate the matched odds ratio (OR) for a change of 10 units in the man concentration of *S atra* in the homes of the patients compared with the homes of the controls. Mean concentration of *S atra* is a continuous predictor in the logistic model. It follows that the slope coefficient for *S atra* gives the change in log OR for an increase of 1 unit in mean concentration. Since this was unlikely to be of interest biologically, we decided to consider a change of 10 units in man concentration of *S atra*.[12] To test for interaction with environmental tobacco smoke, a multivariate logistic model was constructed that also controlled for the matching.

fants to live in a house with a history of water damage from roof or plumbing leaks or flooding.[4]

The geographic clustering of the patients' homes. the tory of recurrent bleeding, and the history of water nage in the homes led us to hypothesize that the infants had been exposed to a toxic substance in their homes. We found no differences between patients and controls with respect to exposures to household pesticides, infant are products. or cocaine. After ruling out all other known causes of pulmonary hemorrhage (including coagulopathies and abuse), the findingofhemolysis on peripheral blood smears led us to consider the possibility that the infants had been exposed to toxins produced by indoor molds. Specifically, our a priori hypothesis was



*From Montaña et al.[4]



*CFU/m³ indicates colony-forming units per cubic meter of air. Findings are averaged across all media; mean of means is not the total mean.
†Includes all other fungi observed.

that infants with pulmonary hemorrhage were more likely than controls to live in homes where *Stachybotrys atra* was present. This fungus is known to grow in water-damaged homes[1] and to have toxins that produce hemorrhagic disease and hemolysis in animals[1].

RESULTS

DESCRIPTION OF ILLNESS

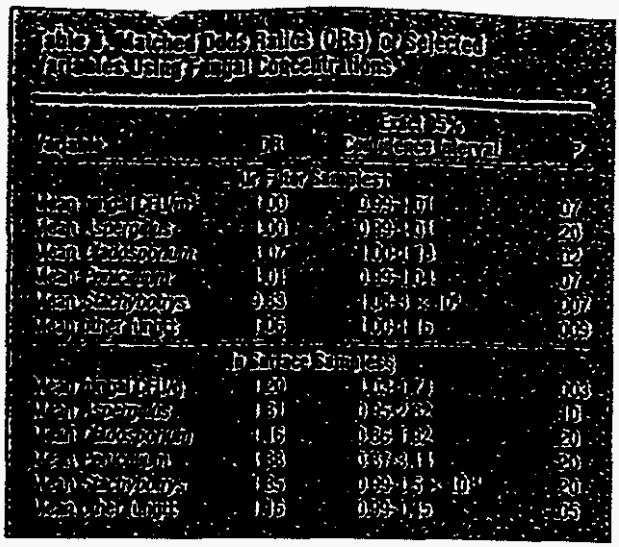
Acute pulmonary hemorrhage occurred in infants who were previously in excellent health. Parents or caregivers noted that the infant abruptly stopped crying, became limp and pale, and then coughed up blood, started grunting, and stopped breathing (Table 1).[4]

COMPARABILITY OF *GROUPS*

Patients and controls appeared to be from relatively comparable socioeconomic settings. For example, mothers of 80% of patients and 83% of controls were receiving Medicaid assistance. Mean maternal ages (21.2 vs 24.3 years). mean maternal education (11.4 vs 11.5 years). and use of an air conditioner (25% vs 29%) were also comparable in both groups.[1]

DESCRIPTION OF HOMES

We were able to gain entry to homes of 9 of the 10 patients and 28 of 30 controls. Patients lived in homes that were an average age of 76 years (range. 59-89 years), whereas controls lived in homes that were an average age of 75 years (range. 35-95 years).



*CFU/m³ indicates colony-forming units per cubic meter of air sampled.
†The OR for a change of 10 units in the variable.
‡Includes all other fungi observed.
§The OR for a change of 1 million units in the variable.

AIRBORNE CONIDIA

Microscopic analyses of dust for airborne *S atra* spores detected *S atra* spores in homes of 7 of 9 patients vs 9 of 28 controls.

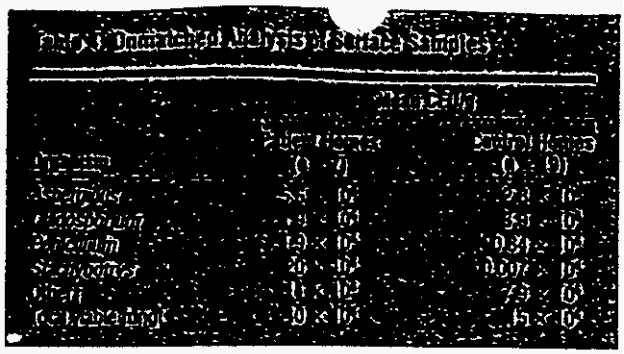
VIABLE AIRBORNE FUNGI

The relative concentrations of the various categories of fungi in filter cassette samples from homes of patients and conuols are shown in Table 2. An unmatched analysis shows that mean CFU counts lor all fungi averaged 29 227 CFU/m³ in homes of patients vs 707 CFU/m³ in those of controls. The mean concentration of *S atra* was 43 CFU/m³ in homes of patients and 4 CFU/m³ in homes of controls when averaged across all media. *Stachybotrys atra* was detected in filter cassette samples from homes of 5 of 9 patients vs 4 of 27 controls. The matched OR for a change of 10 units in the mean concenmtion of *S atra* on the filters was 9.83 (exact 95% confidence interval [CI], 1.08-3 × 10⁶). In other words, if there was a 10-CFU/m³ increase in the concenmtion of *S atra* in the air the infant breathed then the infant was 9.83 times more likely to be a patient (Table 3).

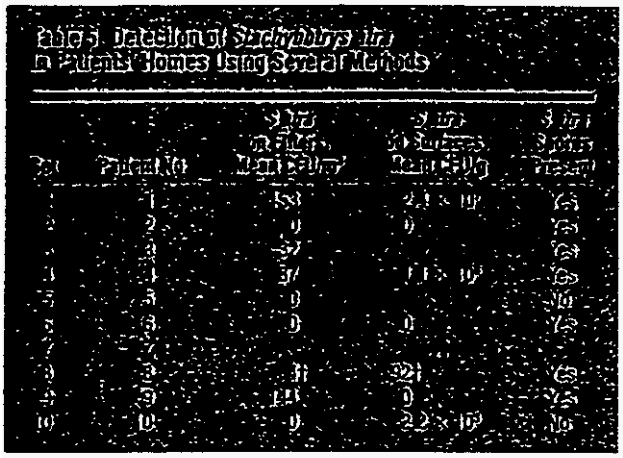
To test for interaction with environmental tobacco smoke, a multivariate matched analysis assessed the impact of *S atra* concentration and exposure to environmental tobacco smoke and showed an OR of 21 (95% CI, 1.07-7.5× 10⁴) for an increase of 10 units in the mean concentration of *S atra* in the presence of environmental tobacco smoke.

SURFACE SAMPLES

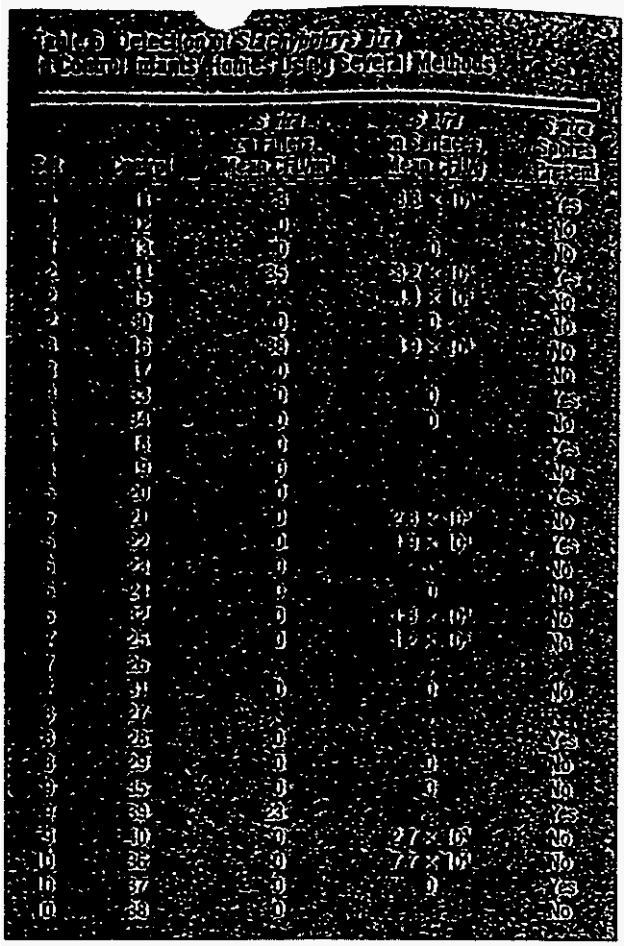
The relative concentrations of the various categories of fungi in surface samples from homes of patients and controls= shown in Table 4. An unmatched analysis shows that the mean concentration of *S atra* was 20 × 10⁶ CFU/g



*Data are averaged across all media. Mean of means is not the total mean. CFU indicates colony-forming units.
†Indicates all other fungi observed.



*Set designates the matched set to which each patient belongs. CFU/m³ indicates colony-forming units per cubic meter of air; ellipses, no sample obtained for analysis.
†Half the limit of detection was used when detected but too few to count (divided by the number of plates).



*Set designates the matched set to which each patient belongs CFU/m³ indicates colony-forming units per cubic meter of air; ellipses, no sample obtained for analysis.

and 0.007 × 10^6 CFU/g in homes of patients and controls, respectively. *Stachybotrys atra* was found in excess of 10^8 CFU/g in surface samples in homes of some patients. *Stachybotrys atra* was detected in surface samples from homes of 4 of 7 patients vs 10 of 19 controls (Table 5 and Table 6). The matched OR for a change of 1 million units in the mean surface concentration of *S atra* was 1.35 (exact 95% CI, 0.99-1.5 × 10^{14}).

Homes of only 1 of the 9 patients failed to reveal the presence of *S atra* using at least 1 of these detection methods, in contrast to those of 12 of 28 controls (OR, 4.9 [95% CI, 0.5-255.6]) (P = .26).

COMMENT

The results of our study suggest that infants with acute pulmonary hemorrhage were more likely than controls to live in homes that had molds, including *S atra*, in the air. The spores of *S atra* contain potent mycotoxins, and we hypothesize that pulmonary hemorrhage occurred after the infants inhaled these spores.

A unique set of circumstances in Cleveland may explain, in part, why this cluster of cases of pulmonary hemorrhage occurred there. The neighborhood in which most of the patients resided consisted of older homes, some in poor repair. Roof and plumbing leaks and flooding with standing water in many of basements were commonly reported, resulting in conditions suitable for the growth of a variety of fungi, including toxigenic *S atra*. Many of the forced-air heating systems of these homes were designed so that return air for the furnaces was pulled from the basements. Because of limited resources, the patients' caregivers reported that waterdamaged items were not removed from the homes.

The numbers of CFU per cubic meter of air sampled for all categories of fungi studied were consistently higher in homes of patients than those of controls when the air samples were collected using filter cassettes (Table 2), suggesting that conditions in these homes favored exceptional levels of fungal contamination.

In some homes, we were able to culture *S atra* from the air but did not find it on surfaces. This may be because the fungus was growing in areas not visible to the investigators, such as behind wallpaper or inside walk. In other homes, we cultured *S atra* from surfaces but did not find it in the air. This may be because the fungus, which is slimy and not easily aerosolized, was not in the air at the time of our sampling. Aerosolization may be an intermittent phenomenon.

Although *S atra* was [illegible] on surfaces in homes of 10 controls. we think that it may be necessary to aerosolize spores to put an infant at risk for pulmonary hemorrhage. The presence of this fungus on surfaces therefore may not be clinically relevant unless it is disturbed or becomes aerosolized.

Stachybotrys atra requires water-saturated, cellulose-based materials for growth in buildings.[7] Its spores contain a variety of toxins,[13] including the most potent members of a large family of mycotoxins called trichothecenes.[14] Two specific trichothecenes produced by *Stachybotrys*, satratoxins G and H, are among the most potent protein synthesis inhibitors known."

A study of the toxigenic potential of strains of *S atra* from the homes of Cleveland patients grown in the laboratory in pure culture on rice demonstrated that these isolates of *S atra* produced satratoxins G and H and a variety of other trichothecene mycotoxins.[16]

Species of the genera *Aspergillus* and *Penicillium* were abundant in the homes studied. which suggests the possibility that metabolites of *S atra* and of other fungi may be present together. Some of these species are also known to produce mycotoxins. eg, *Aspergillus ochraceus, Aspergillus versicolor,* Pmicillium *aurantiogriseum*, and *Penicillium chrysogenum*.[17] However, the matched ORs in Table 3 demonstrate that, in our study, there were no differences in concentrations of *Aspergillus* or *Penicillium* between patient and control homes.

In animals, exposure to trichothecenes *has* been associated with hemorrhaging *and* anemia, but this has *not* been reported previously in human infants

Young or immature animals are more susceptible to the toxic effects of trichothecenes than adults. and hemorrhage and karyorrhexis are conspicuous in rapidly dividing cells.[18] It is possible that very young infants may be unusually susceptible because their lungs are growing rapidly. Conceptually, local inhibition of protein synthesis during the formation of the endothelial basement membrane is likely to lead to capillary fragility and subsequently to stress hemorrhage. Male animals may be more susceptible to these mycotoxins than female animals."

In an earlier report from this investigation, exposure to environmental tobacco smoke appeared to increase the risk for acute pulmonary hemorrhage Nine (90%) of the 10 patients were exposed to tobacco smoke in the home. whereas 16 (53%) of 30 controls were so "posed. In a matched analysis, exposure to tobacco smoke in the home showed an OR of 7.9 (95% CI, 0.9-70.6).[4] Although no association between pulmonary hemosiderosis and environmental tobacco smoke exposure has been reported previously. idiopathic hemosiderosis has been linked to active smoking[20] in a 15-year-old boy. Secondary stressors such as tobacco smoke or other illnesses may play an important role in triggering oven pulmonary hemorrhage.

A variety of investigators have described the effects of exposure to *S atra* among adults.[21,22] Bloody nasal discharge has been documented among adults with occupational exposure to *S atra*. Forgacs and Carll[6] described "severe pharyngitis, or burning sensation in the nose accompanied by bloody nasal discharge and a moderate to severe cough' in workers in whom illness developed after inhaling dusts from *Stachybotrys*-contaminated straw.

In animals, exposure to trichothecenes has been associated with hemorrhaging and anemia,[23] but this & not been reported previously in human infants. In mature mice. studies of intranasal administration of *S atra* spores demonstrated severe alveolar and interstitial inflammation with hemorrhagic exudate in the alveoli.[24,25] Studies of the effects of inhalation exposure of another trichothecene. a biological warfare agent called T-2 toxin. have been made in several animal models. The effects of inhalation were noted to be much greater (>10 times) than those of intravenous exposure.[26]

Stachybotrys atra is thought to be uncommon in North American homes. A study in California found about 3% of 70 homes to have this fungus.[27] A study of 52 homes in eastern Canada found *S atra* in 1home.[28] A recent Canadian study surveyed 401 single-family homes in Wallaceberg, a largely rural community of 12000 in southern Ontario. during the winter of 1994. Approximately *280* species of molds were recovered from dust samples collected in the living areas of the homes. *Stachybotrys* was found in 3 homes.[29] Thus. in large surveys of residential environments. *Stachybotrys* has not been listed among the most common fungi found indoors.

There are several *limitations* of our study. Home sampling for fungi occurred after the infants' hemorrhages, and the conditions at the time of sampling may not have reflected conditions during development of the hemorrhage. However. the fact that none of the patients' parents reported clean-up of water damage suggests the presence of long-standing mold problems. In previous studies. the concentrations of fungi in the air of residences was shown to differ considerably from week to week.[30]

Since each home was sampled only once, it is possible that we may have misclassified some homes as negative for *S atra* when in fact they were positive. *Air* spore counts are known to increase with construction work and vacuuming of carpets.[30] It is well known that spores released in 1 part of a home a n rapidly spread throughout the home on air currents.[31] Since the *sampling* was performed with the environmental hygienists unaware of case status, however. any misclassification would have made an association between the presence of *S atra* and infant pulmonary hemorrhage less likely. Another limitation is that we cannot rule out association of pulmonary hemorrhage with exposure to other toxigenic fungi that we did not uniformly speciate and quantify.

Further research is needed to determine whether this association is causal. Although the association meets several of the epidemiologic criteria for causation (ie, strength of the association, specificity. biologic plausibility, and coherence), other criteria (ie, temporality and consistency with other studies) have not yet been fulfilled.[32] Additional research is needed to determine whether exposure to toxigenic fungi such as *S atra* is associated with acute pulmonary hemorrhage *in* infants in other areas. Such work would be aided by the development of methods to detect spores of *Stachybotrys* or trichothecene metabolites in human tissue.

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The use of trade nam[illegible] commercial products is for identification only and does not imply endorsement by the US public Health Service or the US Department of Health and Human Services or recommendation for use.

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Inflammatory response after inhalation of bacterial endotoxin assessed by the induced sputum technique

Jörgen Thorn, Ragnar Rylander

Abstract

Background—Organic dusts may cause inflammation in the airways. This study was performed to assess the usefulness of the induced sputum technique for evaluating the presence of airways inflammation using inhaled endotoxin (lipopolysaccharide) as the inducer of inflammation.

Methods—To characterise the inflammatory response after inhalation of endotoxin, 21 healthy subjects inhaled 40 µg lipopolysaccharide and were examined before and 24 hours after exposure. Examinations consisted of a questionnaire for symptoms, spirometric testing, blood sampling, and collection of induced sputum using hypertonic saline. Eleven of the subjects inhaled hypertonic saline without endotoxin exposure as controls. Cell counts, eosinophilic cationic protein (ECP), and myeloperoxidase (MPO) were determined in blood and sputum.

Results—A significantly higher proportion of subjects reported respiratory and general symptoms after endotoxin inhalation. MPO and the number of neutrophils in the blood were higher and spirometric values were decreased after the lipopolysaccharide challenge. In the sputum MPO, ECP, and the numbers of neutrophils and lymphocytes were higher after the lipopolysaccharide challenge. No significant differences were found after the inhalation of hypertonic saline compared with before, except for a significantly lower number of lymphocytes in the sputum.

Conclusions—The results support previous studies that inhaled endotoxin causes an inflammation at the exposure site itself, as well as general effects. Sampling of sputum seems to be a useful tool for assessing the presence of airways inflammation, and the inhalation of hypertonic saline used to induce sputum did not significantly interfere with the results found after inhalation of lipopolysaccharide.

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Keywords endotoxin; airways inflammation; inflammatory markers

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There is increasing evidence that diseases and symptoms caused by organic dusts are mainly of an inflammatory nature. A previous analysis of the symptoms prsent after exposure to cotton dust (byssinosis) revealed characteristics related to an inflammation in the airways such as *dry* cough, narrowing of the airways after an acute exposure, and increased airway responsiveness.[1]

Among the many agents present in organic dusts there is substantial evidence to suggest that bacterial endotoxin is a major causative agent. Several symptoms observed after exposure to organic dust have been related to the content of endotoxin in a dose-response fashion, and a relation between the amount of endotoxin in grain dust and airway responsiveness has been reported.[2] A relationship has been found between humidifiers contaminated with Gram negative bacteria/endotoxin and inhalation fever (toxic pneumonitis).[3] Guidelines for exposure to airborne endotoxin in the environment have been suggested.[4]

Of particular interest for causality are studies in which the pure agent it administered to human subjects. For ethical reasons, such exposures are limited to single inhalations. Previous investigations in which healthy subjects inhaled pure endotoxin (lipopolysaccharide, LPS) showed a decrease in forced expiratory volume in one second (FEV_1) and an increase in reported symptoms such as chest tightness and airway irritation, fever, headache, joint and muscle pains, nausea and tiredness after the inhaladon.[5] A decrease in carbon monoxide transfer factor after inhaladon of LPS has been reported,[6] as well as increased airway responsiveness among asthmatic subjects.[7] The cellular inflammatory response after inhalation of LPS was studied in bronchoalveolar lavage fluid where an increase in neutrophils, lymphocytes and fibronectin was reported,[8] and in blood where an increase in the number of neutrophils and C reactive protein (CRP) was observed.[9] An increase in the numbers of neutrophils and lymphocytes, and in levels of myeloperoxidase (MPO) and eosinophilic cationic protein (ECP) has been found in induced sputum after inhalation of LPS.[10]

The purpose of this study was to assess the inflammatory response after LPS inhalation and to compare the effect in two different compartments, blood and induced sputum. The induced sputum technique requires inhalation of hypertonic saline. As this could theoretically influence the response to LPS, a separate experiment on the effects of inhaled hypertonic saline was performed. Respiratory and general symptoms were assessed using a questionnaire.

Methods

SUBJECTS

The study group comprised 21 healthy subjects (nine men) recruited from students at the local university. The mean age was 25 years (range 20–30 years). Potential subjects were interviewed using a standard questionnaire and those accepted in the study were never smokers, had no current colds, no self-reported allergy, no chronic bronchitis or physician diagnosed asthma. They were also non-atopic as defined by a negative reaction in the Phadiatop rest."

STUDY DESIGN

The subjects inhaled 40 μg LPS and were examined before the exposure and 24 hours afterwards. Examinations consisted of spirometric tests and collection of blood and induced sputum samples. Differential cell counts, ECP, and MPO were determined in blood and sputum. A questionnaire was used to identify symptoms.

Of the 21 subjects, 11 (three men) also inhaled hypertonic saline (5%) alone as used in the induced sputum technique. They were also examined before the exposure and 24 hours afterwards. The washout period between LPS inhalation and inhalation of hypertonic saline alone was three months.

The study was approved by the ethics committee of rhc Faculty of Medicine in Gothenburg.

INHALATION OF ENDOTOXIN

Endotoxin (*Escherichia coli* LPS 026:B6 Difco) was suspended in saline and aerosolised using a Pari Boy nebuliser wirh automatic dosing. The nebuliser was calibrated and the automatic output adjusted to give 4 μl per dose. The subject exhaled, placed the nebuliser in the mouth, and started to inhale. Each subject inhaled 20 puffs of endotoxin (500 μg/ml), up to a total of 40 μg.

DIFFERENTIAL CELL COUNTS IN BLOOD

Blood samples were collected for serum measurements and differential cell counts in blood using standard techniques. The results were expressed as 10' cells/l blood.

INDUCED SPUTUM

Sputum induction was performed sccording to the method of Pin *et al*[2] with some modifications. Five percent saline was nebulised ruing an ultrasonic nebuliser (Fisoneb, Fisons, France) on the maximum setting and was inhaled initially for 10 minutes and then in five minute periods over a period of 20 minuter. Ten minutes after rhe start of nebulisation and every five minutes thereafter, subjects interrupted the inhalation of hypertonic saline and were asked to rinse their mouth and throat carefully and to try to cough sputum into a sterile plastic container. The nebulisation was discontinued after 20 minutes. The total amount of hypertonic saline administered was 20 ml.

For safety reasons, spirometric tests were before and immediately after sputum induction, using standard techniques, and the highest values for FEV, and forced vital capacity (FVC) were registered and compared with predicted values.[11] If the FEV, decreased by more than 10% from the baseline value after sputum induction, a β_2 agonist was given by inhalation. One subject could nor perform the spirometric tests after the challenge with LPS owing to her study schedule.

The volume of the collected sputum was determined and an equal volume of Sputasol (Oxoid; Unipath LTD, Basingstoke, Hampshire, UK) was added to obtain a final concentration of 323 mM dithiothreitol (DTT), concentration was used in the first five samples and then decreased to 3.25 mM DTT to increase the number of intact cells. The samples were incubated in a shaking water bath at 37°C for 5–10 minutes to ensure complete homogenisation. The samples were transferred into tubes and centrifuged at 600*g* for five minutes. The supernatants were aspirated and frozen at −70°C for later determination of ECP and MPO concentrations. The cell pellets were washed twice in a washing solution (Hank's salt solution + 5% feral calf serum + 2% EDTA, adjusted to pH 7.2). The total cell count and the cell viability were determined using a Bürker :chamber and trypan blue exclusion. The cell sampler were cytocentrifuged and two investigators each counted at least 200 non-squamous cells on each slide fixed with methanol and stained with May-Grünwald-Giemsa. A cell sample was considered adequate if, on differential cell counting, it contained less than 50% squamous epithelial cells. The results were expressed as 10' cells/l sputum.

In the LPS inhaladon experiment five samples containing more than 50% squamous epithelial cells were excluded from the analyses. A further five samples were excluded for technical reasons (see above).

INFLAMMATORY MARKERS

ECP was assayed in serum and sputum samples by a fluorescent enzyme immunoassay technique (CAP ECP FEIA, Pharmacia Diagnostics AB, Uppsala, Swedm) and expressed as μg/l. Prior to the analyses, cell free sputum samples were treated wirh equal volumes of 0.4% ceryltrimethylammonium bromide (CTAB; Calbiochem) diluted in 0.25% phosphate buffered saline and incubated at room temperature for one hour. After centrifugation at 1500*g* for 10 minutes the supernatants were aspirated and used for ECP determinations. CTAB was used to neutralise the electrical forces in the ECP in order to optimise the FEIA analyses.

MPO was asssyed in serum and sputum samples by a radioimmunoassay technique (CAP MPO RIA, Pharmacia Diagnostics AB, Uppsala, Sweden) and expressed as μg/l.

QUESTIONNAIRE

The subjects were interviewed using a slightly modified standard questionnaire for the assessment of organic dust induced effects.[11] The questionnaire contained questions on cough (dry or with phlegm), chest tightness, shortness

Table 1 Mean (SD) values of inflammatory markers and numbers of cells (×10⁹/l) in blood before and 24 hours after inhalation of 40 µg lipopolysaccharide (LPS) or hypertonic saline

		Before	After	Difference (95% CI)
LPS:				
		n = 21	n = 21	
ECP (µg/l)		7.2 (4.6)	8.3 (4.0)	−1.2 (−3.2 to 0.9)
MPO (µg/l)		208 (75)	331 (147)***	−123 (−182 to −69)
Eosinophils	n	0.19 (0.10)	0.15 (0.09)	0.04 (0 to 0.01)
	%	2.8	1.8**	1.0 (0.4 to 1.6)
Lymphocytes	n	2.87 (0.86)	2.69 (0.95)	0.2 (−0.4 to 0.8)
	%	41.6	27.3***	14.2 (10.4 to 18.0)
Monocytes	n	0.45 (0.30)	0.44 (0.23)	0.01 (−0.10 to 0.12)
	%	6.2	4.5**	17 (0.6 to 2.8)
Neutrophils	n	3.47 (1.25)	6.79 (2.31)***	−3.3 (−4.3 to −23)
	%	49.4	66.7***	−17.2 (−21.3 to −13.2)
Hypertonic saline:				
		n = 11	n = 11	
ECP (µg/l)		9.1 (5.5)	8.8 (4.6)	0.3 (−0.8 to 1.5)
MPO (µg/l)		204 (100)	201 (71)	3 (−30 to 36)
Eosinophils	n	0.17 (0.08)	0.18 (0.101	−0.01 (−0.06 to 0.05)
	%	2 9	3.0	−0.07 (−1.0 to 0.8)
Lymphocytes	n	2.58 (0.75)	2.36 (0.60)	0.2 (−0.2 to 0.1)
	%	41.7	39.6	2.2 (−2.8 to 7.1)
Monocytes	n	0.30 (0.19)	0.29 (0.161	0.01 (−0.09 to 0.11)
	%	4.P	4.8	0.1 (−1.4 to 1.0
Neutrophils	n	3.10 (0.83)	3.30 (1.37)	−0.2 (−1.1 to 0.7)
	%	50.6	52.8	− 22 (−7.6 to 3.3)

ECP = eosinophilic cationic protein; MPO = myeloperoxidase.
**p<0.01; ***p<0.001.

of breath, irritation in the eyes, nose or throat, and nose congestion and itchy nose. Questions were also posed on joint pains, muscle pains, headache, unusual tiredness, skin problems, nausea, and diarrhoea.

ANALYSIS OP DATA

The differences between effect variables *before* and 24 hours *after* the exposure were analysed using non-parametric tests (Wilcoxon's and McNemar's tests) and paired samples tests. The relationship between continuous variables was analysed using a bivariate correlation test (Spearman). Differences were considered statistically significant at a level of p<0.05.

Results

INFLAMMATORY MARKERS AND CELLS IN BLOOD

The results of the determinations of markers for inflammation and cells in the blood are shown in table 1. The amount of MPO was significantly greater after the inhalation of LPS than before. The amount of ECP was slightly greater after the inhalation but the difference was not statistically significant. The number of neutrophils was significantly higher after the inhalation of LPS than before. There was a significant relationship between levels of ECP and MPO before ($r_s = 0.54$; p = 0.01) but not after inhalation of LPS ($r_s = 0.04$; p = 0.85). A statistically significant relationship was observed between the difference (before—after inhalation of LPS) in the number of neutrophils and the difference in MPO levels (r_s = 0.74; p<0.001). The ratio of MPO to neutrophils was significantly decreased after the inhaladon of LPS compared with before (49 versus 64 µg/neutrophil, p = 0.002).

Inhalation of hypertonic saline alone *did* not induce any significant changes in the inflammatory markers or in the numbers of different cells in the blood.

INFLAMMATORY MARKERS AND CELLS IN INDUCED SPUTUM

The results of the determinations of markers for inflammation and cells in induced sputum are shown in table 2. The amounts of ECP and MPO were significantly greater after the inhalation of LPS than before. The increase in MPO was almost threefold compared with the increase in blood of about 50%. The numbers of neutrophils and lymphocytes were significantly higher after the inhalation of LPS. There was a significant relationship between the difference (before—after inhalation of LPS) in levels of ECP and MPO (r_s = 0.80; p = 0.003). There was also a significant relationship between MPO levels and the number of neutrophils after the inhalation of LPS (r_s = 0.80; p = 0.003). The ratio of MPO to neutrophils was significantly decreased after the inhalation of LPS compared with before (1738 versus 3340 µg/neutrophil, p = 0.03).

There were no significant differences 24 hours after the inhalation of hypertonic saline alone, compared with the first occasion, except for the number of lymphocytes which was significantly decreased. With regard to the amounts of MPO and ECP, the standard deviations were high and there were two subjects and one subject, respectively, with much higher values both before and after the inhalation compared with the others. Analyses performed on nine and 10 subjects for amounts of MPO and ECP, respectively, showed no significant differences 24 hours after inhalation

Table 2 Mean (SD) amount of inflammatory markers and number of cells (×10⁹/l) in induced sputum before and 24 hours after inhalation of 40 µg lipopolysaccharide (LPS) or hypertonic saline

		LPS		Hypertonic saline		Difference between the changes induced by LPS and hypertonic saline (95% CI)
		Before	After	Before	After	
n		11	11	11	11	
ECP (µg/l)		79 (55)	212 (187)**	161 (259)	166 (184)	123 (−11 to 267)
MPO (µg/l)		866 (670)	2123 (1512)**	2474 (4333)	2010 (3317)	1721 (−821 to 4264)*
Eosinophils	n	0.01 (0.01)	0.01 (0.02)	0.01 (0.01)	0.01 (0.01)	0 (−0.01 to 0.02)
	%	0.6	0.6	0.6	0.8	−0.2 (−1.2 to 0.9)
Lymphocytes	n	0.04 (0.03)	0.09 (0.07)*	0.07 (0.03)	0 04 (0.02)*	0.07 (0.04 to 0.1)**
	%	3.5	4.1	4 2	2 S**	1.9 (0 9 to 3.0)**
Macrophages	n	0.95 (0.53)	0.84 (0.38)	0 99 (0.40)	0 65 (0.24)	0.1 (−0 3 to 0.5)
	%	71.7	40.4**	58.5	50.9	−23.6 (−36.5 to −10.7)**
Neutrophils	n	0.34 (0.39)	1.38 (1.15)**	0.61 (0.47)	0.72 (0.53)	0.9 (0.3 to 1.6)**
	%	21.6	53.3**	34.6	43.6	22.7 (9 0 to 36 4)**
Respiratory epithelial cells	n	0 04 (0 02)	0 04 (0.02)	0.03 (0 02)	0.03 (0.01)	0.01 (0 to 0.03)
	%	3 2	2.0*	2.1	2.1	−1 2 (−2.4 to 0 06)

ECP = eosinophilic cationic protein; MPO = myeloperoxidase.

	Before	After	Difference (95% CI)
LPS:			
n	20	20	
FEV_1 (l)	4.04 (0.92)	3.97 (0.77)*	0.07 (0.02 to 0.12)
FEV_1 (% predicted)	105 (10)	103 (11)**	1.8 (0.7 to 2.9)
FVC (l)	4.81 (1.02)	4.72 (0.97)**	0.09 (0.04 to 0.14)
FVC (% predicted)	106 (13)	104 (13)**	2 (0.9 to 3.0)
FEV_1/FVC (%)	84 (5)	84 (5)	0.2 (−0.5 to 0.9)
Hypertonic saline:			
n	11	11	
FEV_1 (l)	3.63 (0.60)	3.62 (0.54)	0.01 (−0.06 to 0.08)
FEV_1 (% predicted)	102 (13)	102 (13)	0.3 (−1.7 to 2.2)
FVC (l)	4.27 (0.52)	4.27 (0.50)	0 (−0.08 to 0.08)
FVC (% predicted)	102 (12)	102 (12)	−0.09 (−2.3 to 2.1)
FEV_1/FVC (%)	85 (7)	85 (7)	0.2 (−1.2 to 1.6)

FEV_1 = forced expiratory volume in one second; FVC = forced vital capacity.
*p<0.05, **p<0.01

Table 4 Symptoms (%) reported by 21 subjects before and 24 hours after inhalation of 40 μg lipopolysaccharide (LPS)

	Before (n = 21)	After (n = 21)
Breathlessness	0	39*
Irritation in the eyes	5	10
Irritation in the throat	5	38*
Hoarseness	0	24
Irritation in the nose	5	14
Cough with phlegm	5	14
Dry cough	0	33*
Chest tightness	0	10
Headache	24	62**
Heaviness in the head	10	71**
Unusual tiredness	0	57***
Nausea	0	19
Diarrhoea	0	10
Joint pains	0	24
Muscle pains	10	29
Skin rashes	5	5

*p<0.05; **p<0.01; ***p<0.001.

compared with the first occasion, bur the standard deviations were lower and the values at the first occasion were similar to those found before the LPS inhalation (data not shown).

There were significant differences between the LPS and hypertonic saline inhalations in terms of differences (before—after inhalations) in MPO levels, the numbers of lymphocytes and neutrophils, and a borderline difference for ECP levels ($p = 0.057$) in all subjects.

SPIROMETRIC TESTS

The results of the determinations of FEV, and FVC are shown in table 3. FEV_1 and FVC values were significantly lower 24 hours after inhalauon of LPS than before. Inhalation of hypertonic saline alone had no effect on FEV_1 nod FVC values. For safety reasons, spirometric tests were performed immediately after sputum induction. The FEV_1 and FVC values were then significantly lower than before inhalation on all occasions of sputum induction (data nor shown).

QUESTIONNAIRE DATA

The symptoms reporred by the study subjects before and after inhalation of LPS are shown in table 4. Irritation in the throat, dry cough, breathlessness, unusual tiredness, headache, and heaviness in the head occurred significantly more frequently after the inhalation of LPS than before. In almost every subject in [illegible] the symptoms developed within hours and persisted fur 6–8 hours.

There were relationships bonween MPO levels in the serum and joint pains and unusual tiredness after the inhalation of LPS ($r_s = 0.43$; $p = 0.05$ and $r_s = 0.43$; $P = 0.05$, respectively).

Inhalation of hypertonic saline alone did not cause any increase in the number of reported Symptoms (data not shown).

Discussion

The study included only a small number of subjects, bur each subject was investigated before and after the inhalation of endotoxin and hypertonic saline, acting as their own controls. This increased the possibility of finding exposure related effects. The time after exposure (24 hours) when blood and induced sputum samples were collected was chosen on the basis of results from previous experiments. An increased level of CRP in blood has been reported 24 hours after exposure in subjects challenged with LPS.[9] In animal experiments the invasion of neutrophils in the airways peaks at 24 hours after exposure to endotoxin.[15] The dose of LPS used in this study was chosen as it corresponds to the suggested threshold for inducing clinical symptoms,[1] while 20 μg of inhaled LPS was not found to induce any symptoms.[1]

The results demonstrated several effects after the inhalation of LPS in terms of inflammatory markers and cells, spirometric valuer, and symptoms. The amounts of MPO and ECP in the sputum were significantly increased after challenge wirh LPS. There were also relationships between MPO and ECP levels in the sputum. This suggests the presence of an acute inflammation in the airways, involving borh neutrophils and eosinophils.

Increased levels of ECP and MPO in induced sputum have been found in previous studies of subjects exposed to LPS[16] and in other forms of airways inflammation. Increased levels of: ECP and MPO were found in bronchial lavage fluid samples from patients with chronic bronchitis.[16] Higher concentrariom of ECP in rhe sputum of symptomatic asthmatic patients have been reponcd,[17] as well BY a correlation between spurum levels of ECP and airflow obstruction.[18] Increased amounts of ECP and MPO in serum compared with a control group were recently reported in a work environment with organic dusts.[19] This suggests that ECP and MPO arc markers of a non-specific inflammation.

The number of neutrophils was increased after the challenge with LPS, both in blood and in sputum. These results are similar to those previously reporred by Michel *et al.*[10] The ratio of MPO to neutrophils was decreased after inhalation of LPS, suggesting that an increase in the number of neutrophils does not necessarily cause a corresponding increase in MPO.

The mechanisms of action may be explained as follows. After inhaladon LPS induces a series of intracellular changes in macrophages that can be collectively referred to as activation.

This activation results in the production of chemotactic factors. It has been shown that the neutrophil invasion in the lung results from the secretion of chemotactic agents by alveolar macrophages.[26] Endotoxin induced activation of macrophages also results in increased production of lysosomal enzymes and the production of different cytokines such as interleukin 1 (IL-1) and tumour necrosis factor α (TNF-α), as well as increased production of oxygen metabolites such as superoxide anion, $O_2^{\cdot-}$.[27]

Beyond exerting effects in the exposure site itself—that is, the lung—inhaled LPS may influence the central nervous system. This was shown in the study by general symptoms in terms of fatigue, headache, and heaviness in rhe head. Fever, chills, and malaise can bc caused by bacterial endotoxins or dusts containing endotoxin.[11] The fever causing effect of LPS is thought to bc mediated by TNF-α activity on the hypothalamus, and LPS can cause scrimdon of cerebral catecholsmine and serotonin metabolism.[14] TNF-α has been Found in blood after LPS inhalation.[11] The mechanism responsible for systemic effects after LPS inhalation might be the secretion of TNF-α or other mediators from activated macrophages in the lung.

A major goal of the study was to investigate the effects of LPS in the airways and to determine whether the inhalation of hypertonic saline as required in rhc induced sputum technique influenced the results after LPS inhalation. For ethical reasons we did not use a double blind design, in order to be able to supervise the subjects appropriately after the exposures. For the end points used (spirometric data, cells and inflammatory markers in blood and induced sputum) it is unlikely that *thr* absence of a double blind npproach could have influenced the results. With regard to symptoms, there is obviously some danger of an underestimation of the reported symptoms after inhaladon of hypcrronic saline alone as well as an overestimation after LPS inhalation bur, on the other hand, the symptoms were classic and have been described in previous studies.[1]

To assess inflammation in the airways, bronchoalveolar lavage can bc used as in previous studies[4] but it represents an invasive and complicated clinical procedure, not suitable for field conditions or for investigating a larger number of subjects. The induced sputum technique represents a compmmire. Tie spu—— induction technique with inhalation of hyperconic saline has been used by other researchers, and reproducible results of cellular and soluble markers have been obtained within six days[34] and two days.[35] Significant differences in the inflammatory response after inhalation of LPS compared with saline have previously been reported.[10]

Five samples containing more than 50% squamous epithelial cells were excluded from the analyses, which is not unusually high when working with induced sputum. In this study the DTT was decreased to 3.25 mM the number of acceptable samples increased and was also high in another recently published study (85%).[36] This shows that the technique is valid.

Theoretically, the hypertonic saline used to induce sputum could by itself cause effects. In this study no significant differences (except for the significantly lower number of lymphocytes in sputum) were found for spirometric data, inflammatory markers and cells in blood and induced sputum 34 hours after inhalation of hypertonic saline alone. A recent study evaluaced the effects of repeated sputum inductions within 24 hours and found that the sputum differential neutrophil counr and level of ECP increased significandy from the first to the second induction in 10 normal subjects and 19 subjects with asthma.[19] Another recent study reported a significant increase in the percentage of neutrophils, but nor in the absolute neutrophil counts, .in sputum 24 hours after sputum induction in eight normal subjects.[37] The total amount of hypertonic saline administered in these two studies was 51 ml and 67.5 ml. In our study no significant effects were found 24 hours after inhalation of hypertonic saline alone, bur the total amount of hypertonic saline administered was 20 ml. It is likely that the magnitude of the effect of sputum induction is related to the dose of hypertonic saline given.

The numbers of lymphocytes in sputum were significandy increased after inhalation of LPS and significandy decreased after inhalation of hypertonic saline alone. The reason for the decrease in the number of lymphocytes after hypertonic saline inhalation is not known at present.

In conclusion, the results show that inhalation of LPS causes an acute airways inflammation as indicated by increased amounts of inflammatory markers in sputum and an increase in the prevalence of respiratory symptom. It also causes general effects as shown by an increase in the prevalence of general symptoms ond changes in inflammatory markers in the blood. The changes in cells and inflammatory markers were more pronounced in the sputum than in the blood, which suggests that sputum is more relevant for assessing the presence of airways inflammation after inhalation of endotoxin or organic dusts conraining endotoxin. The inhalation of hypertonic saline used to induce sputum did not significantly interfere with the results found after LPS inhalation.

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Continually Measured Fungal Profiles in Sick Building Syndrome

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Abstract. Buildings with indoor air quality (IAQ) complaints frequently have high airborne concentrations of ***Penicillium*** species, while buildings with few IAQ complaints have an indoor air (IDA) fungal ecology similar to outdoor air (ODA), where ***Cladosporium*** species is usually the dominant microorganism, These studies compared fungal air profiles, measured continually over 6 h in a documented sick building, in IDA in a room experiencing IAQ problems with fungal profiles measured concurrently in ODA. The dominant species collected at both sites were ***Penicillium*** species, ***Cladosporium*** species, and ***Alternaria*** species. In the IDA, *Penicillium* species were always the dominant organisms, ranging from 150 to 567 cfu/m^3 (89.8–100% of the total fungi). In the ODA, ***Cladosporium*** species were dominant in four samples (40.0—70.6%). while ***Penicillium*** species were dominant (52.7-79.6%) in two. These data demonstrate that, even though ODA fungal profiles are changing continuously, IDA fungal profiles in "sick" buildings tend to remain unchanged.

Sick building syndrome (SBS) is a commonly used term for symptoms resulting from indoor air quality (IAQ) problems. Complaints common to SBS include allergic rhinitis, headaches, flu-like symptoms, watering of eyes, and difficulty in breathing [14]. SBS has been recognized for a quarter of a century; the first offcial study of this phenomenon, which examined more than one structure, was published in 1984 [9].

SBS has proven difficult to understand, and no one cause has been described [12]. Early researchers felt that most of the causes of SBS included higher than normal levels of known respiratory irritants. These included such compounds as hydrocarbons, nitrogen, and sulfur dioxides [15], chemicals being released by new buildings and their materials, or known or suspected carcinogens such as formaldehyde, asbestos, radon, and tobacco smoke [21].

It has recently come to the attention of the scientific community that fungi and their spores are associated with IAQ problems [2–4, 7, 8, 16, 18, 20]. Also, fungal contamination in indoor environments has been shown to produce allergies in occupants of these buildings [4, 8, 16, 18.201. We have recently demonstrated that there is a correlation between the prevalence of certain fungi and SBS [6]. In that study, we showed that ***Penicillium*** and ***Stachybotrys*** species appear to be associated with SBS.

The above findings prompted us to initiate a study, in which we attempted to answer two important questions regarding IAQ. The first was, when taking an indoor air sample, is that sample an accurate reflection of the air in that building or is it just a "snapshot" that changes immediately after the "picture" is taken? The second question, a variation of the first, was, do "sick buildings" stay "sick" over an extended period of time or do they "get better" and then become "sick" again?

Methods and Materials

Fungal isolation and identification. Measurements were made in September of 1997 in a multi-story hotel located in the Southwestern United States. The building had a history of staff and occupant complaints typical of IAQ problems. including eye irritation. irritation of the mucous membranes of the nose and throat. lethargy, and headache. On several occasions an IAQ company. called to investigate the building in response to these complaints, reported musty odors, fungal infestation in the heating, ventilation, and air conditioning (HVAC) system, and degradation of the facilities in many rooms by visible fungal growth, and they classified the structure as a "sick building." Additional follow-up studies showed the fungal infestation to be widespread throughout the building. Our research team was granted access to one room experiencing IAQ problems in the building for a 6-h investigatory period. *The* room, a typical hotel room, showed visible fungal stains on the walls.

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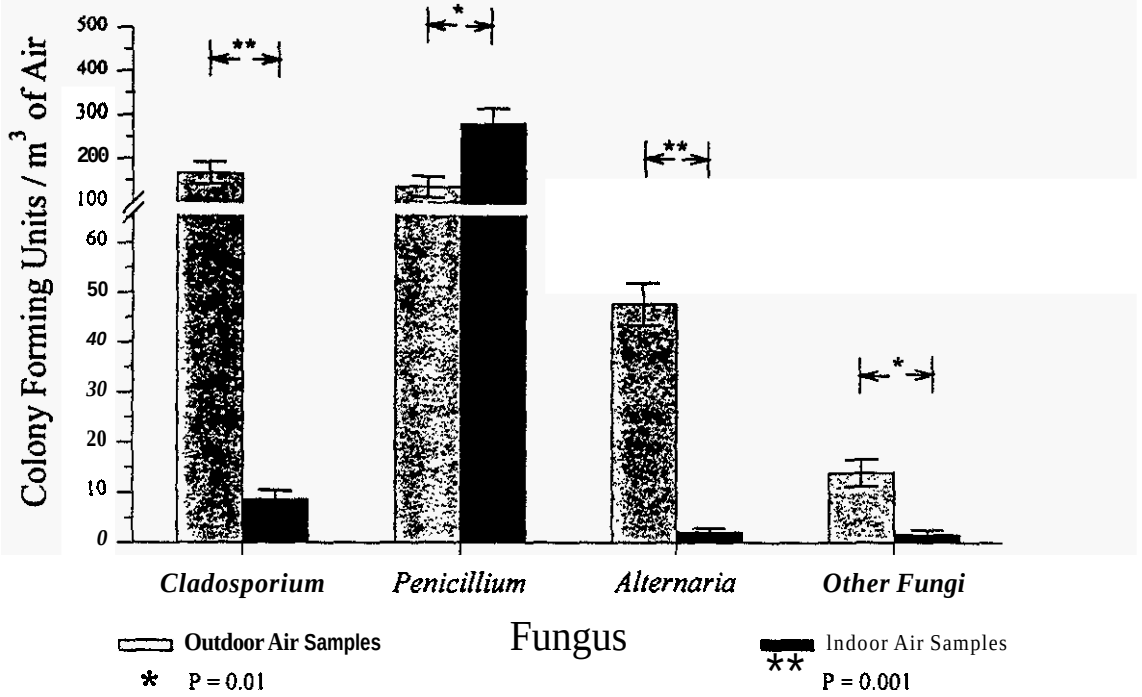

Fig. 1. Fungal concentrations measured in indoor air and outdoor air. Values are mean ± SEM. Each bar represents the average of all samples

During the study. outside air temperature and relative humidity were 21°–28°C and 62–74%, respectively. Inside air temperature and relative humidity were constant at 22°C and 69%. respectively. Air samples (non-aggressive) were drawn in the room, and ODA samples were taken simultaneously on an outside balcony. immediately adjacent to this room. The glass sliding door separating the balcony from the room was always kept closed during air sampling. Air samples were drawn for 5 min in triplicate by means of a two-stage bioaerosol sampler (Model 2000, Andersen Samplers Inc., Atlanta, GA). During sampling, the Andersen samplers were placed approximately 3 ft above the floor level. Sampler were drawn at a calibrated flow rate of 28.4 L/min for 5 min on Sabouraud dextrose agar (SDA), pH 5.6. at 1000, 1100, 1200, 1300. 1400. and 1500 h inside and immediately outside the building. Burge and coworkers [5] have shown that. among the various fungal isolation media. SDA recovers the broadest range and the highest number of airborne fungal species. The agar plates were hand-carried to the laboratory. The plates were incubated for up to 14 days at 22°C and 90% relative humidity (RH). The isolated fungi were identified by standard methods [11, 13]. CFU/m³ were calculated with the formula:

$$CFU/m^3 = [CFU/[(\text{min sampled})\ (ft^3/\text{min})]]/[35.3\ ft^3/m^3]$$

The total fungal CFU/m³ for each air sample was calculated and the ratio for each organism per sample determined. The results were entered according to the area that was sampled (the ODA or the IDA areas). and the average CFU/m³ and the ratio. in terms of percentage, for each organism was determined for each area examined.

Statistics. Values far bath the ODA and IDA were averaged for the six time points. A one-way analysis of variance (ANOVA) was used to test the significance of differences in the ODA and IDA measurements of *Cladosporium*, *Penicillium*, *Alternaria*, and other fungi. A *P* value of less than 0.05 was the minimal level of significance [10]. Fungal concentrations are reported as the mean ± SEM.

Results and Discussion

The dominant fungi collected from the ODA were *Penicillium* species (*chrysogenum*), *Cladosporium* species (*cladosporioides*), and *Alternaria* species (Fig. l). The concentrations of *Cladosporium* species (*cladosporioides*), *Penicillium* species (*chrysogenum*), and *Alternaria* species in IDA were significantly different from the ODA concentrations of these same organisms. Although the concentrations varied with time, *Penicillium* species were always the dominant organisms in the "sick" room, ranging from 150 to 567 cfu/m³ (Fig. 2). The three-plate range for the outdoor air counts for a single collection was from 227 CFU to 535 CFU, whereas, the three-plate range for the indoor air counts at a single collection was from 156 CFU to 585 CFU. These values for *Penicillium* species represented from 89.8% to 100% of the total fungi in the IDA.

In the ODA over the same time period. *Cladosporium* species were dominant (40.0–70.6%) in four of the 6-h samples (Fig. 3), ranging from 47 to 378 cfu/m³. In the two other ODA samples, *Penicillium* species were dominant (52.7–79.6%). ranging from 45 to 299 cfu/m³. A comparison of the temporal variation of the ODA and IDA fungal profiles (Figs. 2 and 3) shows that the ODA profiles vary with time and that changes occurring in the ODA have little effect on the fungal profile of the IDA.

These data demonstrate that, while fungal profiles in

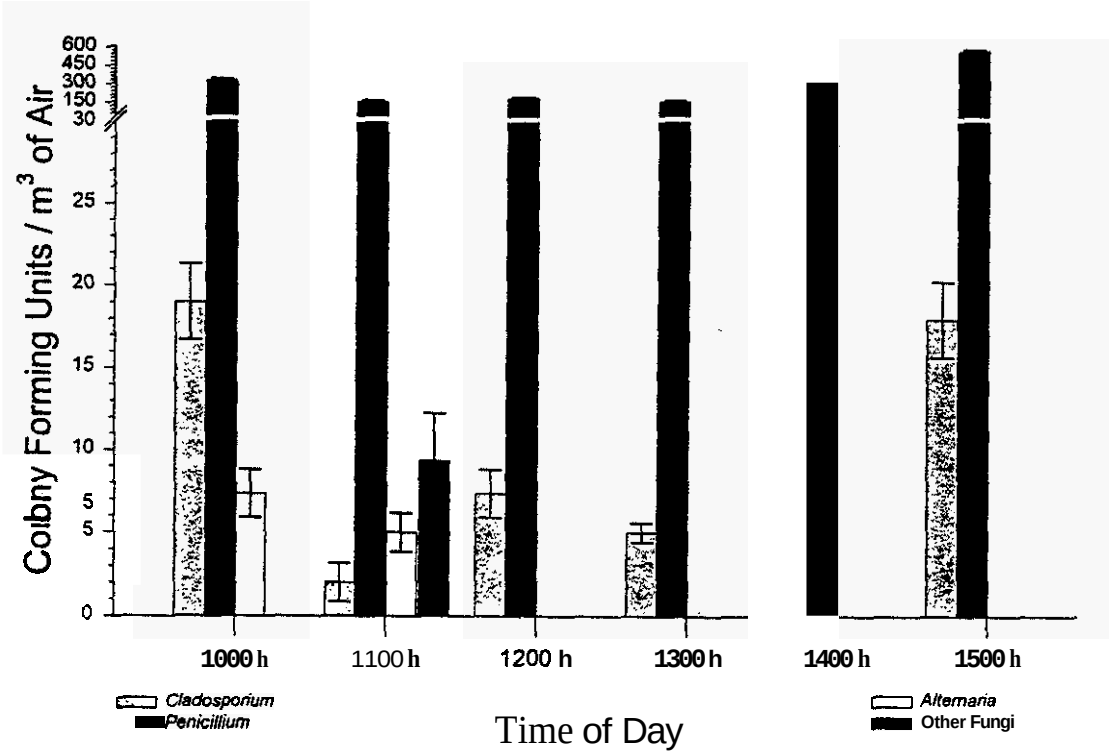

Fig. 2. Fungal profiles measured in indoor air. Values are mean ± SEM. Each bar represents the mean of three samples.

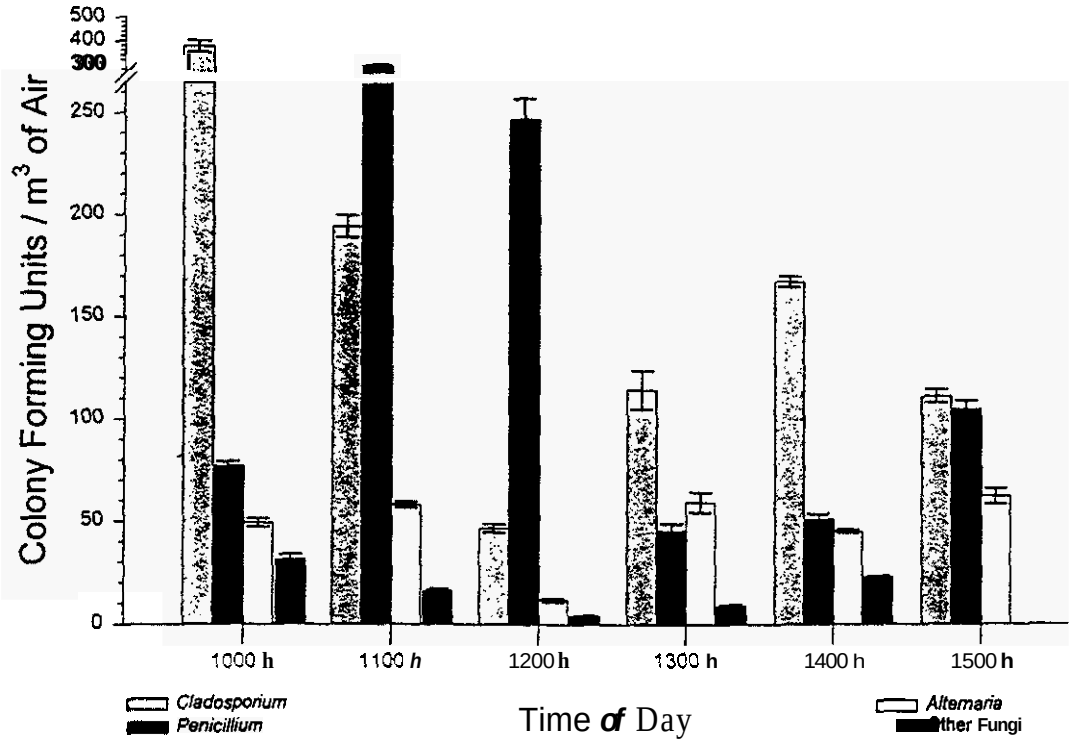

Fig. 3. Fungal profiles measured in outdoor air. Values are mean ± SEM. Each bar represents the mean of three samples.

ODA are continually changing, fungal profiles in "sick" building IDA arc independent of fungal profiles in ODA. Moreover, "sick" buildings tend to stay "sick" for extended periods of time, in part because of a stasis in the IDA fungal profile. These results are in accord with our earlier study [6] of public schools located in various areas throughout the United States.

Other workers have documented the imponance of Penicillium species colonization of buildings and its relationship to occupant complaints. Aheam and coworkers [2] demonstrated that the air-handling units and fiberglass duct liner of the HVAC system may be extensively colonized by Penicillium species and *Cladosporium* species, even in buildings without a history of water damage. Moreover, rooms supplied by an extensively colonized HVAC system may give rise not only to elevated concentrations of fungi, but fungal parts and metabolites, including volatile organics [1].

In conclusion, these results indicate that occupant complaints in sick buildings that we have studied have been associated with Penicillium species and that these sick buildings tend to remain sick (for at least 6 h) despite changes in the outdoor fungal ecology profile. Moreover, these results indicate that single measurements of fungal profiles in indoor air ("snapshots") may provide useful information in a building assessment.

ACKNOWLEDGMENTS

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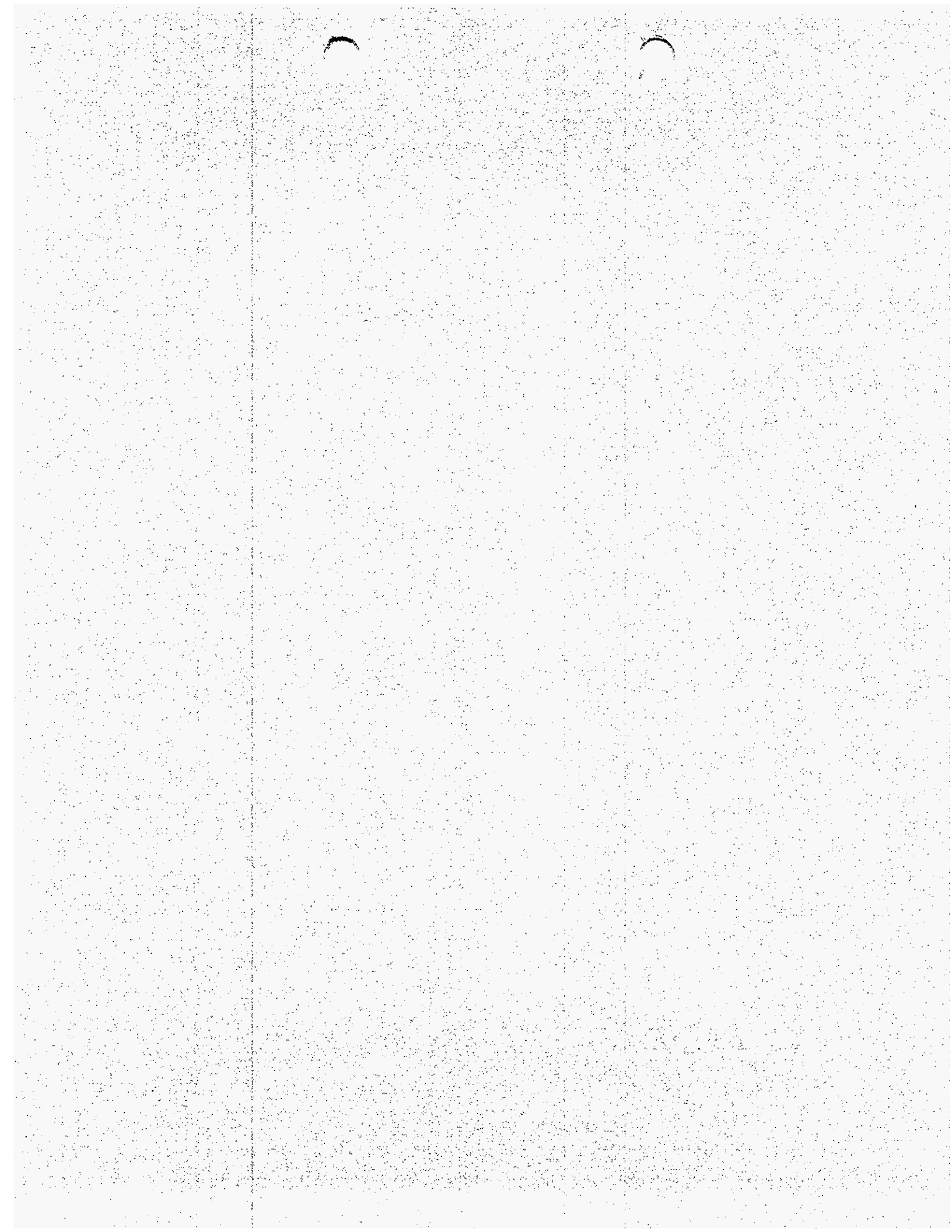

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Highly Sensitive Protein Translation Assay for Trichothecene Toxicity in Airborne Particulates: Comparison with Cytotoxicity Assays

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Screening assays for environmental mycotoxins in bulk samples currently use cytotoxicity in cell cultures, bul their application to air particulate samples often lacks sensitivity and specificily for fungal spores. An assay based on inhibition of protein synthesis using translation of firefly luciferase in a rabbit reticulocyte system has been developed for the detection of trichothecene mycotoxins found in the spores of loxigenic fungi. Ethanol extracts of air particulates trapped on polycarbonate filters are ultrafiltered and applied at several dilutions to a translation reaction mixture. The activity of translated luciferase is measured directly in a luminometer, eliminating the need for radioisolopes and time-consuming sample processing. Parallel standard curves using a commercially available trichothecene provide for expression of the results in T-2 toxin equivalents per cubic meler of air. The assay can be completed in 2 h and is readily applicable lo multiple samples. Comparison to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cytotoxicity assay indicates a 400-fold increase in sensitivity of trichothecene detection in addition to a much higher specificity for these toxins. Initial field testing indicates a strong correlation between the measured level of toxicity and thr presence of toxigenic fungi detected with microbiological methods. In conclusion, this luciferase translation assay offers a rapid and highly sensitive and specific method for quantilative detection of trichothecene mycotoxin activity in air particulate samples.

Because fungal viability may be short-lived compared to toxin stability. methods of detecting toxins or toxicity are much preferred over those requiring fungal culturing. Quantitative tests for airborne environmental fungi which are most widely used are based on culturing of air particulates collected on filters and determination of the number of viable spores. The making of public health decisions would be greatly facilitated by the development of rapid and affordable strategies which provide accurate quantitative assessment of possible environmental exposure to fungal toxins.

Existing methods of trichothecene toxin detection include costly, highly technical approaches. such as gar chromatography or mass spectroscopy (23). Immunodetection requires specific antibodies which are not readily available at the present time (8). Thin-layer chromatography has been used to detect mycotoxins, but its sensitivity is significantly lower than that of cytotoxicity measurements (25). Cell culture-based cytotoxicity assays (12, 14, 19) appear to work well with samples generated under controlled conditions, such as growing fungi on sterile substrates, but interpretation of the results becomes problem. atic when environmental bulk samples are studied. In addition to fungi, those samples are commonly heavily contaminated by bacteria, which raise3 the possibility of various synergistic effects of mycotoxins and other substances, such as endotoxin. Specificity may be less of a problem in the application of cytotoxicity assays to airborne particulates, but the sensitivity of these tests would preclude quantitative evaluation (22). In an attempt to use this approach with airborne particulates, we have tested swine kidney cells by using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (14). We have found that cytotoxicity assays. while yielding reproducible results, lack both the specificity and sensitivity needed to detect mycotoxins in fungal spores collected on filters in indoor air sampling using low, (3 liters/min) to-medium (22 liters/min)-flow pumps. For cytotoxicity assay either high-flow pumps or prolonged collection periods are needed to sample the large quantities of air required.

Mycotoxin detection based on the inhibition of protein synthesis has been described by others (28). Toxin detection and methods used in studying the mechanism of action at the protein translation level (27) have relied on the use of radioisotopes and vary in sensitivity to 12,13-epoxytrichothecenes. Although they can have serious limitations, bioassays based on inhibition of protein synthesis demonstrate high specificity and sensitivity toward trichothecene mycotoxins. We have developed a nonradioactive assay based on translation firefly luciferase in a rabbit reticulocyte system and have compared its sensitivity and specificity with those of the MTT assay by using both pure mycotoxins and air particulate samples collected from fungus-contaminated houses.

MATERIALS AND METHODS

Materials and reagents. PK15 cells were obtained from the American Type culture Collection, Manassas. Va. DON (deoxynivalenol) and T-2 toxin were purchased from Sigma. St. Louis, Mo. Satratoxin G was a generous gift from Bruce Jarvis, University of Maryland. The rabbit reticulocyte lysate, luciferase mRNA, amino acids. magnesium acetate. potassium chloride, recombinant RNase inhibitor, and luciferase array reagent used were from Promega. Madison, Wis. RNase T-1 from *Aspergillus oryzae* was from Gibco BRL. Gaithersburg, Md.

Collection and processing of air samples. Air sampler from fungus-contaminated houses, as well as clean control rooms, were collected on polycarbonate filters (pare size, 0.8 μm; Poretics Corp., Livermore, Calif.) using low-flow (3 liters/min for 24 h) and medium-flow (18 and 22 liters/min for 8 h) pumps. Filters

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were extracted overnight in 10 ml of 95% ethanol and sonicated for 30 min. Another 5 ml of ethanol was added to the filter, and sonication was repeated for 30 min. Extracts were passed through 0.22-μm-pore-size GV Millex (Millipore Corp., Bedford, Mass.) filters to remove particulates and evaporated. Ethanol was the solvent of choice because of its compatibility with the filters used to remove endotoxin and RNase. Dried samples were reconstituted in small volumes of ethanol and appropriately diluted with buffer or culture medium for testing. For cytotoxicity studies, samples were filtered through Ultrasart D20 (Sartorius, Goettingen, Germany) to remove endotoxin. Extracts used in translation inhibition assays were passed through Millipore Ultrafree-MC 5000 NMWL filter units to remove proteins.

Bulk samples and isolated fungal spores. *Stachybotrys chartarum*, originally isolated from a home in Cleveland, Ohio (JS5817; American Type Culture Collection catalog no. 201211), was grown in culture on rice. Rice (100 g) was suspended in 60 ml of distilled water and allowed to stand for 1 to 2 h before autoclaving. The rice was sterilized by autoclaving, inoculated with suspensions of 7-day-old conidia, and incubated at 28°C for 4 weeks. Additional water (5 ml) was added axenically after 48 h of incubation. Cultures were stored at 4°C until needed. Small portions of rice culture (volume not important) were shaken into a small plexiglass chamber (8 by 8 cm [internal dimensions]) provided with two openings. The chamber had previously been disinfected with 70% isopropanol. Incoming air was filtered through sterile glass wool in a 37-mm filter cassette, and the air-entrained spores were collected on an open-faced 37-mm cassette connected to an external vacuum source. The chamber was hand shaken to aerosolize the spores within the chamber, and the entire operation was performed in a chemical fume hood. When the collection filters were completely black, the vacuum was stopped, the chamber was disassembled, and the filters were transferred to sterile 50-ml centrifuge tubes for transport. The operation was repeated until it was no longer possible to collect spores from the rice. Samples from the filters were examined microscopically for the presence of hyphae and/or conidiophores and tested for fungal and bacterial contamination by streaking on malt extract agar and incubation in trypticase soy broth, respectively. Filters containing spores were rinsed with 1 to 2 ml of phosphate-buffered saline. Spores released from the filter were enumerated microscopically with a hemocytometer. Known numbers of spores were then pelleted by centrifugation and extracted with ethanol as described above. Bulk samples (fragments of wallpaper and drywall and of dust, carpet fibers, etc.) were weighed prior to the extraction. Extraction was performed as described for filters.

Identification of fungal species in residences with water problems. Bulk samples collected in residences (surface samples such as dust, wallpaper, etc.) were cultured under standard conditions on potato dextrose agar and Rose Bengal plates for 1 to 3 weeks at 30°C. Fungi were identified based on their morphology.

Cell culture. PK15 porcine kidney cells were cultured at 37°C in Eagle's minimum essential medium with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, Earle's balanced salt solution, and 5% newborn calf serum antibiotic free in an atmosphere of 5% CO_2. For cytotoxicity measurements, cells were trypsinized (0.25% trypsin, 0.03% EDTA) and passaged onto 96-well plates at a density of 5×10^5/ml and a volume of 150 μl/well. After 24 h, confluent cells were exposed to toxins and extracts for 72 h prior to MTT assay.

Preparation and storage of trichothecene mycotoxins and air particulate extracts. Concentrated stocks of T-2 toxin, satratoxin G, and DON (1 mg/ml) were prepared in ethanol to ensure their complete solubility and subsequently diluted in the culture medium used to grow porcine kidney cells as described above just prior to their addition to the cultures. The final concentration of ethanol did not exceed 1% in the culture medium and was kept under 0.05% in the translation reaction mixture. Storage of trichothecenes and filter extracts in aqueous solutions was avoided at all times to prevent the loss of toxin activity observed by others (28).

MTT assay. MTT assays (20) were performed as described by Hanelt et al. (14) by using a Bio-Rad 3550 plate reader.

Translation of firefly luciferase mRNA. The translation reaction was carried out with 33% rabbit reticulocyte lysate, 0.25 mM magnesium acetate, 110 mM potassium acetate, 8.3 ng of luciferase mRNA per μl, 0.33 U of rRNasin RNase inhibitor per μl, 3 μM amino acid mixture, 4 mM dithiothreitol, and diluted toxins or extracts in a final volume of 1 to 20 μl. Using smaller volumes allows one to save reagents but is technically difficult when using manual pipetting. Following incubation at 30°C for 90 min, samples were rapidly frozen on dry ice. The luciferase translation assay has to be performed with great caution to avoid the introduction of RNase from the laboratory environment. We routinely use sterile techniques, sterile glassware and plasticware, and RNase-free water and reagents.

Luciferase activity assay. The luciferase assay was performed following sample thawing and 20-fold dilution with 20 mM Tris/HCl (pH 7.8). Luciferase assay reagent (50 μl) containing 20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 μM coenzyme A, 470 μM luciferin, and 530 μM ATP (pH 7.8) was mixed quickly with 5 μl of the diluted translation mixture and read in an Optocompt I photon-counting luminometer (MGM Instruments Inc.). The activity of all samples was expressed as percent control (water added in place of toxin or extract). A purified luciferase preparation was used to choose the range of light intensity (relative light units [RLU]) proportional to the amount of luciferase present in the sample. Control samples (no toxin added) consistently yielded about 60,000 RLU, corresponding to 2 μg of luciferase per liter.

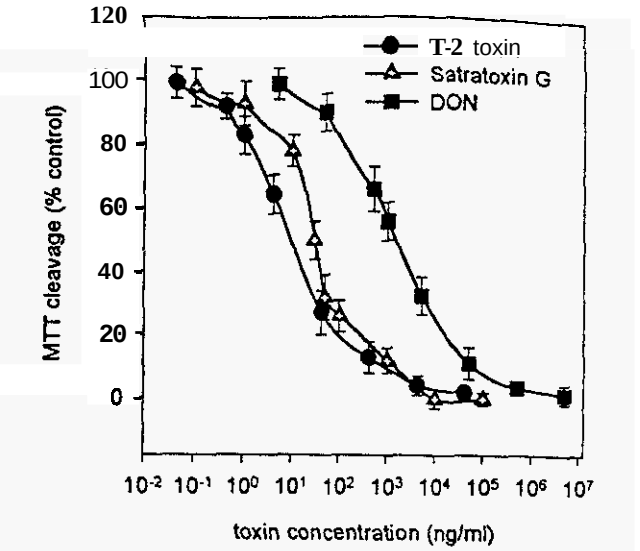

FIG. 1. Effects of T-2 toxin, satratoxin G, and DON on the MTT cleavage activity of PK15 cells. The values are means ± SEM ($n = 8$). T-2 toxin yielded 50% inhibition at 9.14 ng/ml ($r^2 = 0.98$), satratoxin G did so at 29.9 ng/ml ($r^2 = 0.98$), and DON did so at 1,470 ng/ml ($r^2 = 0.98$).

Data analysis. Dose-response curves were plotted and analyzed by using SigmaPlot and TableCurve programs (Jandel Scientific). Data from dose-response experiments were fitted into logistic dose-response equations, and 50% effective concentrations were calculated. Correlation coefficient (r^2) values were used to assess the goodness of fit and ranged from 0.950 to 0.999.

RESULTS

Cytotoxicity assays. Porcine kidney cells are highly susceptible to trichothecene mycotoxins, as demonstrated by Hanelt et al. (14) in a study comparing three different cell lines. We have found that swine kidney PK15 cells were more sensitive to trichothecenes and more resistant to solvents such as methanol and ethanol (no significant changes in MTT cleavage activity were detected at up to 5% ethanol) than the MRC-5 human lung fibroblast cells used by others for cytotoxicity measurements (19). Based on these findings, PK15 cells were chosen for further studies.

Three mycotoxins were tested for their cytotoxic effects on PK15 cells: two simple trichothecenes, T-2 toxin and DON, produced by *Fusarium* sp., which are commercially available, and a macrocyclic trichothecene, satratoxin G, isolated from *S. chartarum* (18). As shown in Fig. 1, T-2 toxin was found to inhibit MTT cleavage by 50% at a concentration of 9.14 ± 0.95 ng/ml. Satratoxin G produced the same effect at about three times the concentration (29.9 ± 2.6 ng/ml). DON was much less potent against PK15 cells, with 50% inhibition at 1.47 ± 0.12 μg/ml.

The cytotoxicity of air particulates collected in several homes with visible mold growth was evaluated by using material equivalent to 0.1 to 4.0 m^3 of air. Under these conditions, the MTT test did not reveal any cytotoxic effects of air particulate extracts (data not shown). Based on the sensitivity of the MTT test (see Table 2), the cytotoxicity of those samples was lower than that of 1 ng of T-2 toxin/m^3.

Bulk samples such as dust, fragments of carpeting, plaster, or wallpaper collected in houses with moisture and fungus problems were demonstrated to contain very high levels of cytotoxicity. While the level of cytotoxicity was significantly

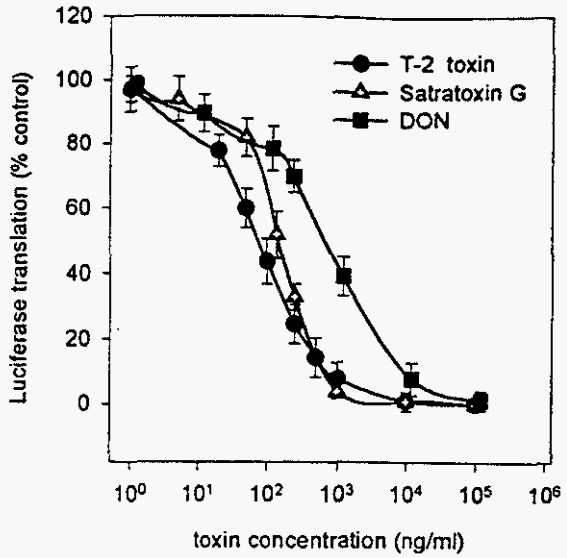

FIG. 2. Effects of T-2 toxin, satratoxin G, and DON on luciferase translation in rabbit reticulocyte lysate. The values shown are means ± SEM (n = 8). T-2 toxin yielded 50% inhibition at 78.5 ng/ml (r^2 = 0.99), satratoxin G did so at 148.5 ng/ml (r^2 = 0.99), and DON did so at 757 ng/ml (r^2 = 0.99).

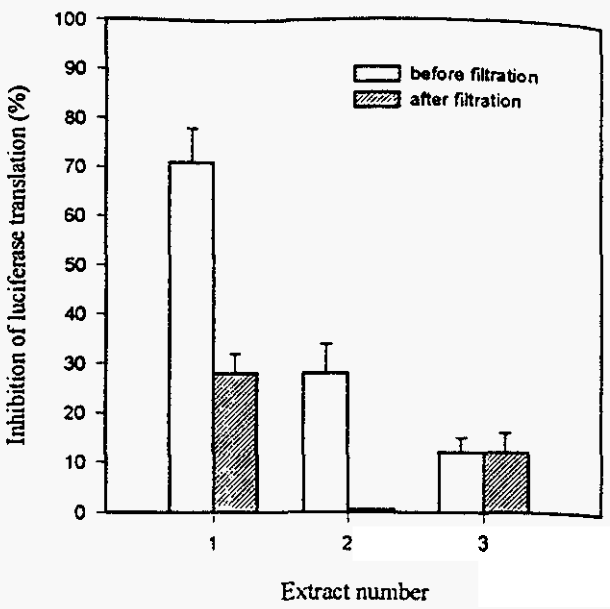

FIG. 3. Effect of filtration through Millipore Ultrafree-MC 5000 NMWL centrifuge filters on the protein translation inhibition activity of three different air particulate sample extracts. The values are means ± SEM (n = 3).

decreased by using filters which exclude bacterial endotoxin, e.g., Ultrasart D20 (data not shown), other toxic agents from paint, glue, or dyes that are highly soluble in ethanol are likely to be present.

Translation inhibition assay. The in vitro luciferase translation system was used to study the effect of trichothecenes on protein translation in a cell-free rabbit reticulocyte system. The standard reaction conditions for rabbit reticulocyte lysate (24) have been modified as described in Materials and Methods to reach high translation reaction efficiency and, at the same time, limit the use of reagents. Figure 2 shows the concentration-dependent inhibition of translation of firefly luciferase mRNA by T-2 toxin, satratoxin G, and DON. Similar to the cell culture-based system, there is a significant difference in the effect of DON (50% inhibition at 757 ± 43 ng/ml), satratoxin G (50% inhibition at 148.5 ± 7.7 ng/ml), and T-2 toxin (50% inhibition at about 78.53 ± 13 ng/ml). Interestingly, T-2 toxin and satratoxin G are much less effective in this system than in PK15 cells.

The air particulate extracts from fungus-contaminated houses were strongly inhibitory in the rabbit reticulocyte system (Table 1). Control sterile filters showed no inhibition of luciferase translation.

The content of extracts remained the main concern with this highly sensitive assay, especially with respect to RNase, which can be a serious problem in translation-based applications. The recombinant inhibitor that inhibits the RNases A, B, and C included in the reaction mixture is not effective against many bacterial and fungal RNases (3).

To determine whether the observed inhibition can be attributed to RNases or trichothecenes, the extracts were filtered through Millipore Ultrafree-MC 5000 NMWL centrifuge filter units with a molecular weight exclusion limit of 5,000. This procedure should remove proteins, most importantly, RNases and proteases. As shown in Fig. 3, such filtration of extracts of environmental air particulates leads to reduction of inhibitory activity. This suggests that these extracts contain significant levels of RNases or other high-molecular-weight compounds that interfere with the translation process or destroy the translation product (proteases). The concern about RNase interference was further investigated by incubating luciferase mRNA (1 μg) with air particulate extracts under conditions identical to those used for translation. Subsequent electrophoresis on 1% agarose (Fig. 4, lane 2) shows that RNA disappears completely following incubation with unfiltered extract. However,

TABLE 1. Toxicity of air particulates collected in residences with moisture problems

House no., room(s)	Fungus cultured	Toxin equivalents (ng/m³)[a]		Vol of air containing particulates causing 50% translation inhibition (m³)
		T-2	SG	
71, bedroom	*Stachybotrys* sp.	1.09	2.06	1.441
71, kitchen	*Stachybotrys* sp.	1.66	3.15	0.941
73, bedroom	*Stachybotrys* sp.	1.75	3.33	0.899
74A, bedroom	*Stachybotrys* sp.	0.98	1.86	1.600
74B, bedroom				
Test 1	NA[b]	0.50	0.94	3.1
Test 2		0.47	0.89	3.3.
Test 3		0.54	1.02	2.900
75, baby's room	*Stachybotrys* sp.	1.10	2.09	1.421
75, toy room	*Stachybotrys* sp.	1.12	2.12	1.395
78, baby's room	*Rhizopus* sp.[c]	1.91	3.64	0.816
78, basement	*Rhizopus* sp.[c]	4.56	8.68	0.342
79A, bedroom	*Stachybotrys* sp.	17.90	34.10	0.087
79B, bedroom	*Stachybotrys* sp.	8.00	15.20	0.195
80, front room	*Stachybotrys* sp.	14.10	27.00	0.110
80, baby's room	*Stachybotrys* sp.	17.30	33.00	0.090
Control rooms (n = 3)	NA	0[d]	0[d]	NA
Control rooms (n = 2)	NA	0.01[d]	0.02[d]	NA
Control room	NA	0.09[d]	0.20[d]	NA

[a] r^2 values for fitted dose-response curves ranged from 0.950 to 0.998. T-2, T-2 toxin; SG, satratoxin G.
[b] NA, not available.
[c] Heavy growth precluded identification of other fungi.
[d] Single-dose measurement.

FIG. 4. Luciferase mRNA (1 μg) incubated with air particulate sample extracts. Lanes 1, control (water added instead of extract); 2, extract showing a high degree of luciferase translation inhibition; 3, the same extract as in lane 1 filtered through a Millipore Ultrafree-MC 5000 NMWL centrifuge filter; 4, 1 μg of standard luciferase mRNA.

incubation with the same extract filtered through Millipore Ultrafree-MC 5000 NMWL units did not lead to detectable degradation of RNA.

In order to quantitate the sensitivity of the translation assay to RNase, we used fungal RNase T_1 from *A. oryzae* (molecular mass, 11 kDa). As shown in Fig. 5, subpicogram amounts of RNase affect the translation of luciferase. Filtration through Millipore Ultrafree-MC 5000 NMWL filters efficiently removes up to almost 2 ng of RNase. Increasing the concentration of RNase results in leaking of enzymatic activity through the filter. To be sure that the extracts were RNase free, they were filtered and assayed a second time by using the amount that reduced the translational activity by about 50% in the first assay. If the inhibitory effect on luciferase translation is reduced following the second filtration, it could be attributed to the presence of RNase in the extract. If observed changes remain within the limits of experimental error (several percent), we conclude that other inhibitors, most likely mycotoxins, were responsible for the inhibition of protein translation. To date, none of the environmental samples have shown reduced inhibition after the second filtration, demonstrating that the single filtration is sufficient to remove low levels of RNase present in the air particulate extracts.

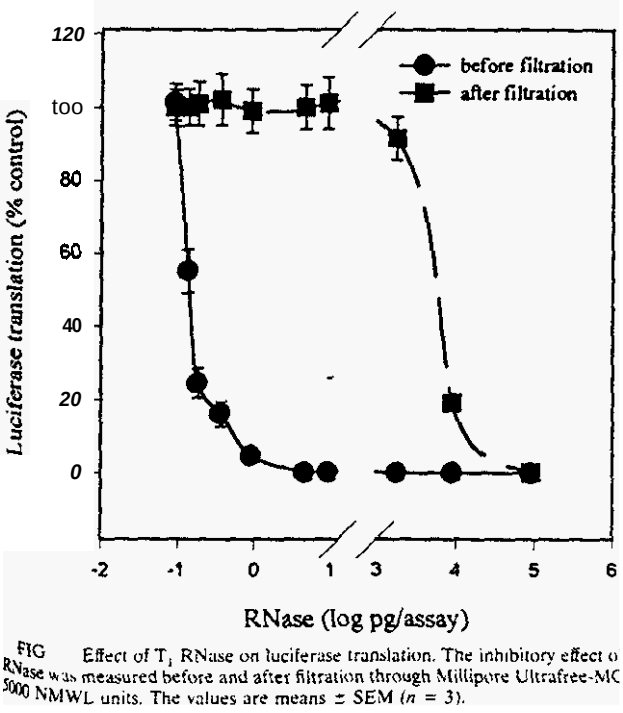

FIG. 5. Effect of T_1 RNase on luciferase translation. The inhibitory effect of RNase was measured before and after filtration through Millipore Ultrafree-MC 5000 NMWL units. The values are means ± SEM (n = 3).

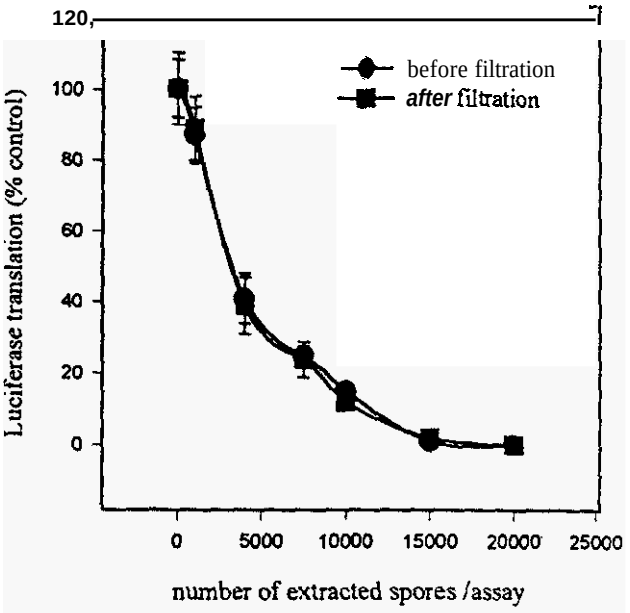

FIG. 6. Effect of *S. chartarum* spore extracts on luciferase translation in the rabbit reticulocyte system. Comparison of extracts filtered through Millipore Ultrafree-MC 5000 NMWL units with unfiltered extracts. The values are means ± SEM (n = 3).

Control experiments performed with pure T-2 toxin indicate that there is no loss of trichothecenes in extracts due to the filtration. T-2 toxin solutions can be passed through Millipore Ultrafree-MC 5000 NMWL units up to three times without any significant change in toxicity as estimated by the luciferase translation assay.

Because the focus of the tests is on fungal spores, it was important to determine if the spores contain detectable amounts of RNase activity. The presence of RNase in fungal spores has been reported (16, 30). *S. chartarum* spores were extracted in accordance with our standard ethanol procedure and used in the luciferase translation assay. The dose-response curves (Fig. 6) generated with extracts obtained before and after filtration through Millipore Ultrafree-MC 5000 NMWL units were virtually superimposable, indicating the lack of RNase activity in spore ethanol extracts. This suggests that either *S. chartarum* spores do not contain RNase activity or none of the enzyme is extracted or active under the conditions of the experiment.

In addition to removing traces of RNases, ultrafiltration would remove other possible interfering agents, such as proteases or endotoxin. We have not been able to detectpny effect of endotoxin on luciferase translation at concentrations of up to 1 μg/ml.

Testing and quantitating the toxicity of environmental **samples.** The luciferase translation method was used to detect and quantitate the toxicity of air particulates collected in houses with known water and mold problems, where the presence of toxigenic mold has been confirmed by culturing of bulk samples. Dose-response curves were generated by using filtered (Millipore Ultrafree-MC 5000 NMWL filters) extracts of polycarbonate filters. Dose-response curves for T-3 toxin or satra-

toxin G were run in parallel with each experiment. The results are expressed as toxin equivalents per cubic meter of air determined by matching the 50% inhibition points of the experimental extract curves and the T-2 toxin and satratoxin G curves. The amounts of T-2 toxin and satratoxin G (nanograms) causing 50% inhibition were equated to the volume of extract (microliters) causing 50% inhibition of the luciferase translation. The volume of extract was then converted to the volume of air sampled (cubic meters), and toxin equivalents (nanograms per cubic meter) were obtained.

Table 1 shows the results of toxicity tests in several houses and rooms. The highest toxicity corresponds to about 17 ng of T-2 toxin or 34 ng of satratoxin G present in 1 m^3 of air. Either control rooms (clean rooms with no evidence of mold) had no detectable toxicity, or their toxicity was no higher than 0.091 ng of T-2 toxin equivalents/m^3 (5 to 200 times lower than that of contaminated rooms). Detecting toxicity of control rooms required using much larger amounts of extracts, corresponding to 5 to 10 m^3 of sampled air. With the routine sampling of residences yielding a maximum of 10 m^3 (8 h at 22 liters/min), only a single-point reading could be obtained.

To further validate the testing procedure, the reproducibility of multiple screening was assessed. Table 1 (house 74B) shows results obtained after collecting air samples in the same room three times for 8 h each time within a period of 72 h. The three separate samplings yielded toxin equivalent values of 0.502, 0.471, and 0.54 (mean, 0.505; standard error of the mean [SEM], 0.02) ng/m^3.

DISCUSSION

The aim was to develop a rapid and inexpensive method to quantitatively assess exposure to trichothecenes as a biomarker for toxigenic fungi such as *S. chartarum*, which has recently been linked to an outbreak of pulmonary hemosiderosis in infants (see reference 10). Currently, airborne exposure to toxigenic fungi can only be estimated based on the results of culturing or spore counting of air particulate samples. Airborne concentrations of detected culturable spores are often falsely low (1, 11). Furthermore, because different isolates of the same fungal species can produce various amounts of mycotoxins, depending on the growth conditions (18, 21), the isolation of a toxigenic fungus from a building cannot be taken as an indication of the level of toxin exposure. Spores that have lost the ability to germinate still contain stable trichothecene mycotoxins. Toxicity tests may confirm both the presence and the toxic potential of fungal isolates in a particular home environment. Thus, measurement of total trichothecene toxicity rather than the number of viable spores is a more accurate approach.

Existing literature on cytotoxic effects of fungal spores collected on polycarbonate filters and pure mycotoxins suggests that cytotoxicity assays may be suitable for evaluation of inhalation exposure to toxigenic fungi (12, 14). Cytotoxicity has been used to measure toxic effects of fungal spores under controlled experimental conditions. Pasanen and coworkers (22) employed the fetal lung cell-based assay to demonstrate toxicity of airborne spores of *S. chartarum* growing in the laboratory on substrates, such as hay, grain, and wallpaper, that have been sterilized prior to fungal contamination. In a study of problem buildings using kidney cells and an MTT test, Gareis (12) demonstrated the cytotoxicity of fungus-contaminated samples of gypsum board. However, the specificity and quantitative aspect of those assays have not been tested in large practical building surveillance studies.

Cytotoxicity experiments employing the MTT assay described in this report demonstrate that porcine kidney (PK15) cells are highly susceptible to pure trichothecene toxins. Similar midpoint toxicity values for T-2 toxin of 2.8, 5.6, and 9.0 ng/ml for melanoma cells, keratinocytes, and hepatoma cells were reported by others using the neutral red assay (2). The MTT assay used with MDBK cells yielded 50% inhibition at 1 to 1.5 ng of T-2 toxin per ml (15). Cytotoxity experiments performed with numerous mycotoxins and a different line of swine kidney cells yielded 0.8 μg of DON per ml and 6.2 μg of satratoxin G per ml for 80% MTT cleavage activity (14).

By using extracts of polycarbonate filters which have been exposed to the air in houses with mold and moisture problems, we were unable to detect any cytotoxic effect on PK15 cells. In contrast, bulk samples collected in parallel with the air samples exhibited very high cytotoxicity. This was expected, since bulk samples, in general, contain much higher concentrations of microbes than can be found in air particulates (11). It seems that at least some of that toxicity could be attributed to the presence of bacterial endotoxin and other high-molecular-weight compounds. In most cases, filtration of ethanol extracts through Ultrasart D20 filters, which removes molecules larger than 20 kDa, led to reduction of the observed cytotoxicity. In summary, we find the MTT cleavage-based cytotoxicity assay to be suitable for screening of highly toxic bulk samples, especially after removal of endotoxin and other high-molecular-weight compounds, but not sufficiently sensitive or specific to detect and quantify the trichothecene toxicity of air particulates. High-flow pumps with impingers collecting much larger samples may help solve the sensitivity problem, but this does not appear to be practical for routine sampling, especially in residential buildings. The lack of specificity toward fungal toxins and the potential for synergistic effects do not appear to be readily resolvable in this system.

The primary mode of trichothecene action in living cells is inhibition of the protein translation process (6). Assays based on protein synthesis have been used to detect and compare mycotoxins, as well as to study their mechanism of action (27, 28). Translation inhibition-based tests performed with cultured cells appear to be highly sensitive to trichothecenes, with T-2 toxin 50% inhibition values of 10 to 15 ng/ml for CHO cells and 1 ng/ml for MDBK cells (15). T-2 toxin has been shown to be much less effective in cell-free translation systems, such as rabbit reticulocyte lysates requiring microgram-per-milliliter concentrations (27). The protein translation assays previously described all involve the use of radioactive amino acids and require several hours to several days to complete the tedious and labor-intensive processing of samples.

In recent years, the translation of firefly luciferase has been used in molecular biology as a nonradioactive alternative to detect and quantitate the expression of reporter genes and as a control for in vitro translation. The luminescence of in vitro-translated luciferase can be easily detected and quantified. Luciferase catalyzes ATP-dependent conversion of luciferin to oxyluciferin with concomitant release of light. The quantum yield of this reaction is the highest in efficiency of any known biological reaction (26). The light emitted from firefly luciferase is directly proportional to the number of luciferase enzyme molecules when the substrate is not in excess (7). Luciferase activity can be measured directly in the translation mixture within seconds. The entire testing procedure, including protein translation, can be completed in less than 2 h. The rabbit reticulocyte system has been extensively studied and optimized to yield functional, biologically active proteins (13) and is currently available from several commercial sources.

We have demonstrated that the tricliothecenes T-2 toxin, satratoxin G, and DON readily inhibit the translation of firefly

TABLE 2. Comparison of trichothecene detection limits obtained by different methods

Toxin and parameter	Luciferase translation assay[a]	MTT cytotoxicity assay[b]	MTT/LT[b]	Cytotoxicity[c,d]	Thin-layer chromatography[e]	Enzyme-linked immunosorbent assay[e,f]
T-2 toxin						
Conc at 80% activity (pg/ml)	20,000	1,000				
Vol used (μl)	0.25 (1)	100				
Amt detected (pg)	5 (20)	100		10–40,000	500,000	1–40
Ratio			5–20			
Satratoxin G						
Concn at 80% activity (pg/ml)	50,000	10,000				
Vol used (μl)	0.25 (1)	100				
Amt detected (pg)	12.5 (50)	1,000		630,000		
Ratio			20–80			
DON						
Conc at 80% activity	200,000	200,000				
Vol used (μl)	0.25 (1)	100				
Amt detected (pg)	50 (200)	20,000		100,000	500,000	250,000
Ratio			100–400			

[a] The value in parentheses is the practical volume that can be accurately dispensed. This volume is four times larger than the amount needed for the luciferase assay and results in an increase in the detection limit (see text).
[b] This report. LT, luciferase translation assay.
[c] Robb and Norval (25).
[d] Hanelt et al. (14).
[e] Dietrich et al. (8).
[f] Casale et al. (5).

luciferase mRNA in a cell-free rabbit reticulocyte system. T-2 toxin and, to a lesser extent, satratoxin G are not as effective in the reticulocyte lysate as they are in PK15 cells. The greater potency in intact cells can be explained by toxicity independent of translational mechanisms such as effect on membranes or additional toxicity of toxin metabolites (4, 29). In the case of DON, only a small difference in potency between PK15 cells and the luciferase translation system was detected. DON contains different specific side groups than T-2 toxin and inhibits both the elongation and termination steps of the protein translation, whereas T-2 toxin inhibits the initiation step (9, 27).

Although the effective toxin concentrations in the luciferase translation assays may be similar to (as in the case of DON) or even higher than (as in the cases of T-2 toxin and satratoxin G) those in the cytotoxicity assay, the practical sensitivity advantage of the luciferase translation assay results from the very small volume of extract that can be used. This is evident from Table 2, which contains a comparison of the detection limits of the luciferase translation method and the MTT cytotoxicity assay described in this report, as well as methods described by others, using cytotoxicity tests, thin-layer chromatographic analysis, and immunodetection. The luciferase translation test is a two-step procedure composed of a protein translation step and a luciferase assay step. Only 0.25 μl of translation mixture is required to obtain readings of about 60,000 RLU. Practically, one is not able to attain this limit in the first step by using regular pipetting, by which only 1 μl of translation mixture can be accurately dispensed. This increases the practical detection limit by a factor of four, hence, the difference between the real and the practical (in parentheses) volumes and amounts detected (Table 2). Use of robotic devices allowing accurate dispensing of submicroliter volumes should close the gap between the real and practical detection limits. The sensitivity of the assay remains comparable to the range of immunodetection. However, unlike immunodetection, the luciferase translation assay does not require an array of specific antibodies and measures combined toxicity rather than concentrations of individual toxins, thus providing a broader assessment of exposure. Toxicity analysis of problem houses finds toxin levels corresponding to nanogram amounts of T-2 toxin and satratoxin G, which is almost 1,000 times more than our practical detection limits. Furthermore, the procedure is conveniently standardized by parallel determination of T-2 toxin and satratoxin G dose-response curves. Although our use of rabbit reticulocytes yields very reproducible results, some batches of lysate occasionally demonstrate slightly different initial activity and altered sensitivity to trichothecenes, especially after prolonged storage. Therefore, using standard toxins provides an additional measure of interexperiment reproducibility and allows the expression of toxicity in terms of T-2 toxin and/or satratoxin G equivalents. The choice of standard toxins is somewhat arbitrary. Initially, T-2 toxin was selected because of its commercial availability and low cost and the relative abundance of available literature. Satratonin G was subsequently included because it was detected in isolates of *S. chartarum* from water-damaged houses in Cleveland, Ohio, included in a study of pulmonary hemosiderosis in infants (IS). However, this toxin is not commercially available, and the literature describing its action is rather scarce (17). Although possible effects of RNases and proteases, as well as other, unidentified substances interfering with protein translation, are potentially serious limitations of the assay, we have largely excluded these problems by the double filtration of extracts to remove enzymes and other large molecules. Exclusion of RNases and certain biomarkers, such as endotoxin, indicative of the presence of gram-negative bacteria allows one to focus on trichothecene mycotoxins as an indicator of exposure to toxigenic fungi.

In addition to its sensitivity, the luciferase translation assay yields highly reproducible results, as demonstrated by multiple screening of the same moisture problem house. As an activity assay, the luciferase translation test does not provide the toxin composition of environmental samples, which can only be in-

vestigated hy using costly multimethod approaches. as described by Andersson et al. (1). Rather. the luciferase translation assay is a practical, rapid. and inexpensive means to detect and quantify the fungus-derived toxicity of air particulates from problem indoor environments. Inclusion of this assay in a battery of fungal tests to assess indoor environments is planned in order to demonstrate its projected utility.

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Toxicity screening of materials from buildings with fungal indoor air quality problems *(Stachybotrys* charfarum)

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Abstract:

Samples of building materials visibly contaminated with moisture-related fungi (drywall, fiberglass, wallpaper, wood) were tested with indirect (FFL) and direct (MTT) cytotoxicity screening tests that are particularly sensitive to ***Stachybofryscharfarum*** toxins. In addition, microscopic, chemical, immunochemical (Roridin A enzyme immunoassay) and mycological culture analyses were performed. In all cases in which building occupants had reported verifiable skin, mucous membrane, respiratory, central nervous system or neuropsychological abnormalities, cytotoxicity was identified. Results of a cytotoxicity screening test of field samples, such as the direct MTT test method, will give investigators of health problems related to indoor air quality problems important toxicity information.

Introduction:

The presence of indoor fungi, such as *Stachybotrys chartarum (a.k.a. S. atra)* on building materials has been recognized as an important risk factor for indoor air contamination. Serious adverse health effects in animals and humans associated with intense indoor or occupational exposure to *S. charfarum* and other fungal species *(A. versicolor, A. fumigatus, Fusarium spp., Trichoderma viride, Penicillium spp.*, etc.) have been described and reviewed elsewhere in detail (1-6). High exposure levels of airborne pathogenic fungi in the work environment have been reported among farmers, wood workers, composting waste workers, hospitals and office workers with defective ventilation systems. In recent medical-epidemiological investigations toxic-inflammatory effects

have been found in infants with fatal hemorrhagicpneumonitis (7-9)or in officeworkers handling moldy paper materials and breathing air contaminated with fungi (10). These effects are thought to be related to toxic metabolites (mycotoxins) produced by certain fungal species, such as those in the genera *Stachybotrys, Aspergillus, Penicillium, Trichoderma* and *Fusarium.* S. chartaium is clinically important because it produces biologically very potent mycotoxins, such as trichothecenes (i.e., Satratoxin) and spirolactones, which interfere with protein synthesis, DNA and cellular or the humoral immune system (11). The production apparently depends on certain environmental conditions and the nature of materials or substrates, such as high water content and cellulose (12).

Conventional industrial hygiene exposure assessment has focused generally on speciation (taxonomy) and quantification of culturable (viable) fungi, but rarely on toxicity analysis. Toxicity screening *tests* have not been available in the past for such investigations. However, such information would be important to improve the understanding of the related pathology and for medical treatment or other intervention strategies.

The goal of our investigation was to evaluate fungal samples taken from water damaged homes of Occupants who reported significant health complaints (sentinel cluster investigation) for the presence of toxicity (i.e., mycotoxins) using indirect and direct cytotoxicity screening *tests.* Some other analytical tests, in addition to conventional bioaerosol sampling, were also done. Other significant sources of indoor air contamination (dust, pesticides, VOCs etc.) were ruled out based on the occupants' information and walk-through visual inspection of the problem homes or offices by a trained professional.

Method

Assessment and documentation of health problems:

Occupants' health complaints and abnormalities related in time and place to occupancy of a water-damaged home or workplace with fungal contamination were verified by an occupational and environmental physician with extensive experience in the assessment of building-related illnesses. A standardized healthsymptom questionnaire was utilized to detect abnormalities of certain target organs, such as upper and lower airways, eyes, skin and mucous membranes, central nervous system and constitutional complaints (flu-like symptoms, loss of appetite, weight-loss). Selected fungal IgE (immediate type allergy) and IgG (delayed typeallergy) antibodies were tested in the building occupants as markers of exposure. Further, lymphocyte enumeration and function tests (T-lymphocyte mitogen proliferation analysis) were done in some cases to detect and verify immune dysfunction and suppression suggestive of fungal exposure. The medical laboratory tests were analyzed by licenced commercial labs following standard quality. assurance (QA) procedures (IBT-laboratory, Kansas, Ms., and Specialty Laboratory, Inc., Santa Monica, Ca.). Only important and supportive laboratory findings will be presented, since this is not the focus of this paper. Asubgroup of individuals underwent formal, standardized neuro-behavioral evaluation by a clinical psychologist (Detailed results will be presented elsewhere).

Fungal sampling:

Culturable fungi were sampled with a conventional impaction air sampler, Andersen N6 (singlestage), with a flow rate of 28.3 l/min using a sampling time of 3 min. Culture

media were malt extracts (MEA) and cornmeal agar (CMA). Bulk samples were evaluated by light or electron microscopy and then cultured.

Cytotoxicity analysis:
Visibly contaminated bulksamples along with clean control samples were collected by the investigator and were analyzed using two different cytotoxity test methods, with known high sensitivity to trichothecenes: **A)** The MTT-cell culture assay and a Swine kidney target cell lines were used as a direct method (using original building materials)(13, 14). The continuous feline fetus lung cell line assay (FFL) was used as an indirect method (fungal cultures were isolated from theoriginal samples and grown on rice-agar in the laboratory under optimal conditions) (15). In addition, a subset ofsamples taken from a problem house was further chemically analyzed utilizing a high performance liquid chromatography (HPLC) method (16) and an enzyme immunoassay which detects macrocyclic trichothecenes such as Roridin A (17, 18). Visibly non-contaminated building materials were collected from the problem buildings and were used as control samples in the analysis.

B) **MTT** cytotoxicity test:
The colorimetric tetrazolium MTT cleavage test has been described as a sensitive bioassay for the evaluation of a series of mycotoxins. It has been used as a standard in thescreening ofa variety of mycotoxin-contaminated materials such as cereals and feed (19). The principle of this bioassay is based on the transformation of the yellow tetrazolium salt MTT by viable, living cells (via mitochondrial dehydrogenase) *to* purple formazans. Swine kidney monolayer cells (SK), known to be sensitive to mycotoxins, were used as target cells. The minimum concentrations of the test reagents to cause toxic effects were determined on the basis of the statistically determined values of 80% cleavage activity. Eight wells of the 96-well tissueculture plate (row 1) remained empty and served as blanks. After decanting the cell culture medium from the microtiter plates, 100 µl of the complete medium. containing 1.7 % ethanol and 0.3 % DMSO (v/v) were added to each well. Aliquots of the sample solutions were evaporated and dissolved in MEM containing 1.7 % ethanol and 0.3 % DMSO (v/v). On separate plates serial log 2 dilutions of the sample solutions were prepared and 100 µl of each dilution were transferred in duplicatesto the cell culture plate. Final concentrations of the crude extract of the samples tested ranged from 3.8 to 500 mg/ml of cell culture medium. All plates were incubated for *20* hours at 37 °C in a humidified atmosphere with *5%* CO_2. A volume of 20 µl each of the MTT stock solution was then added to the wells and plates incubated for another 4 hours. Supernatant was then removed using a multichannel micro pipette and 100 µl DMSO was added to each well in order *to* dissolve the dark formazan crystals. Theoptical density of each well was measured spectrophotometrically with an ELISA-Reader at a wavelength of *540* nm using row 1 as the blank. Mean extinction values and standard deviations of each sample concentration were compared with those *of* the corresponding control and expressed as % cleavage activity in comparison to cell controls (100 %). The statistical significance of the test results was calculated using the students t-test at $p=0.01$. The minimum concentrations of the test reagents to cause toxic effects were determined on the basis of the statistically determined values of 80% cleavage activity. Any cleavage activity (y-axis) below 80% would be considered a "positive" or 'toxic" test result.

B) The Feline Fetus Lung (FFL) cell assay test:
The feline fetus lung cell assay test (FFL) is an indirect method, fungal extract from the

bulk sample are cultured under laboratory conditions and it requires microscopic evaluation of cell death caused by toxic Fungal metabolites. Feline fetus lung cells (FFL) are rapidly growing fibroblast-type cells, which are particularly sensitive to small amounts of trichothecene mycotoxins and relatively easy to work with for the experienced laboratory technician. In our study, *S. chartarum* was isolated from bulk samples and grown on malt extract agar. The *S. chartarum* strains were grown on moist rice (60%) for two weeks at 20-23°C and thereafter for 2 weeks at 6-8°C. The toxins were extracted with methanol-water, filtered and evaporated to dryness. Dry extracts were dissolved in methanol-phosphate-buffered saline and used for the FFL cytotoxicity test (15). A standard cell culture medium was used. The assay was carried out on micro well plates. Diluting the samples allowed an estimation of the amount of toxin present. The cell cultures were incubated in normal cell culture for 5-7 days. The toxic effects were microscopically observed with fewer cells visible compared to the untreated sample. *S. chartarum* strands that were found to be positive in the FFL test were further chemically analyzed as described below.

Chemical analysis:

A subset of samples was chemically analyzed to detect trichothecenes and other selected chemical compounds. The analysis was performed in this fashion: The dark brown-black gummy samples (0.2 - 0.6 g) were dissolved in 1-2 ml of chloroform and applied to a cleanup column of silica gel (2 g, Whatman LPS-1, 20-30 m) packed in hexane. Three Fractions (I, II and III, ca. 15 ml each) were collected: 90% diethyl ether-10% hexane (I); 6% methanol in dichloromethane (II); 100% methanol. (III). The percent recovery was 80-95%. and the general distribution of material in the fractions was ca. 20-30% fraction I, 5-15% fraction II and 70% fraction III. Fractions were dissolved in 1 ml of methanol and analyzed by high performance liquid chromatography (HPLC) under the following conditions (5 µl per injection): Gilson model 302 gradient HPLC with a Knauer Variable Wavelength Monitor set at 260 nm and a Shimadzu C-R3A Chromatopac integrator; Superco C-18 column, 4.6 x 250 mm, solvent system of 60% methanol (MeHO) - 40% water (Containing 5 acetic acid) with a flow rate of 1.2 ml/min under the following retention times: G = 5.3 min; H = 5.7 min. The retention times for the stachybotrylactones and stachybotrylactams are in the t = 11 - 17 min range. The levels of Satratoxin, stachybotrylactones and stachybotrylactams were estimated based on available standard curves (20). For Satratoxin H, levels below 1 µg/sample are not considered reliably detected and are designated ND (not detected). Trace levels are in the 2-5 µg/sample levels; modest levels (+) are in the range of 5 -10 µg/sample, and high levels (++ to +++) are in the range of 20-50 µg/sample. The levels of Satratoxin and stachybotrylactones were estimated from the total areas under the peaks in the 11-17 min retention time range and are considered to be upper limits since there may be other, but minor, contaminating peaks in this retention time area.

Roridin A immunoassay test:

A subset of samples was analyzed using a direct enzyme immunoassay (EIA) based on monoclonal antibodies against roridin A, which has cross-reactivity with satratoxin H and other macrocyclic trichothecenes. The results were compared with those of the MTT tests. An aliquot of the raw extract from the sample was evaporated to dryness. The residue was dissolved in phosphate buffered saline (PBS) containing 10% methanol and directly assayed by EIA using Mab 5G11, verrucarin A horseradish peroxidase conjugate, and a roridin A standard curve. The detection limit of this EIA system was 0.1 ng roridin A/ml.

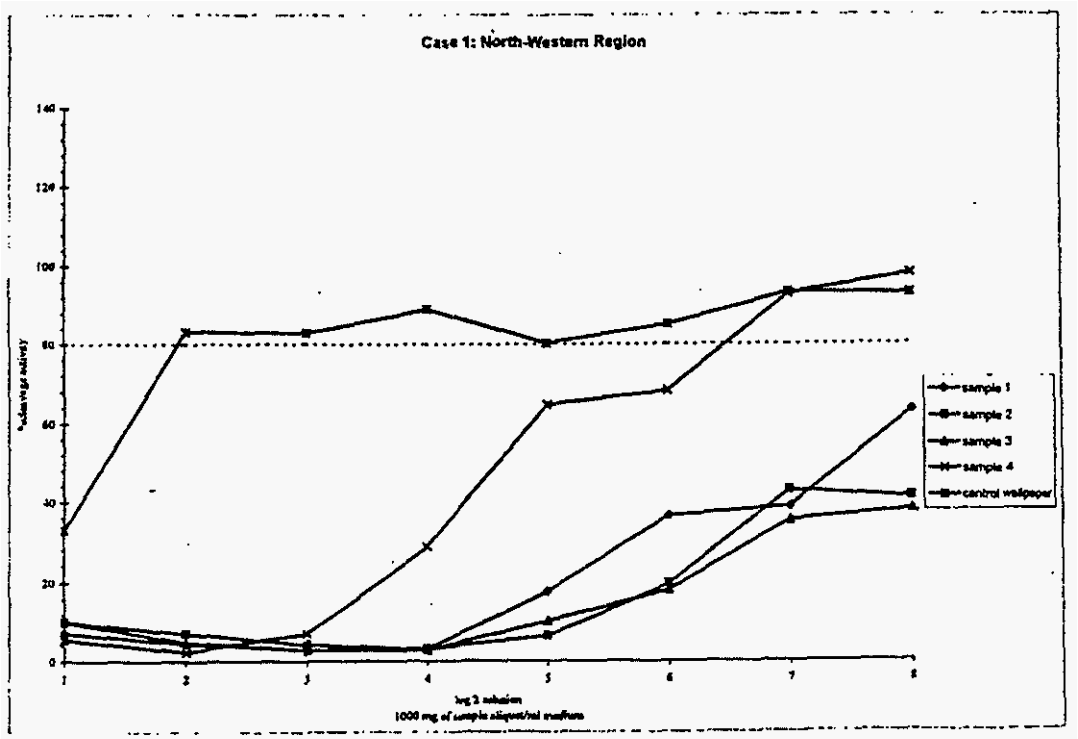
Case histories and sampling results:

Results of environmental sampling; important clinical case histories and cytotoxicity tests for various climatic regions in the United States are presented in the following:

Case 1 Worth-Western region, coastal. moderate temperature and humidity):
S. chartarum was found on an area of approximately 6 - 9 m^2 gypsum board (*drywall") in the crawl space (cellar) beneath an apartment with air leaks into the apartment. The gypsum board paper sheeting was heavily overgrown with black-sooty looking fungal material. Several family members from the above apartment were complaining about burning/tingling sensation of the feet and hands, flu-like symptoms, extreme fatigue, chronic headaches and unusual memory problems and irritability. These symptoms were progressive for several months. Serum IgG antibodies were detected against *A. fumigatus, P. notatum, Trichoderma viride, S. chartarum* (25 mcg/ml). *B* and T lymphocytes were normal, except for a very low percentile of natural killer cells (NK) (2-4 %).

Air and bulk sample analysis:
Air sampling results for culturable fungi were in the range of 447 to > 4,711 CFU/m^3, with a predominance of *S. chartarum, Aspergillus spp., Penicillium spp.* and *Cladosporium*. Gypsum board paper analysis showed a concentration of 4,130,000 CFU/g with a predominance of *S. chartarum, Penicillium, A. versicolor, Ulocladium, Acremonium.*

The MTT cytotoxicity screening test of the bulk samples taken from the crawl room showed high toxicity up to dilution step six to eight compared with the control. (Fig. 1).



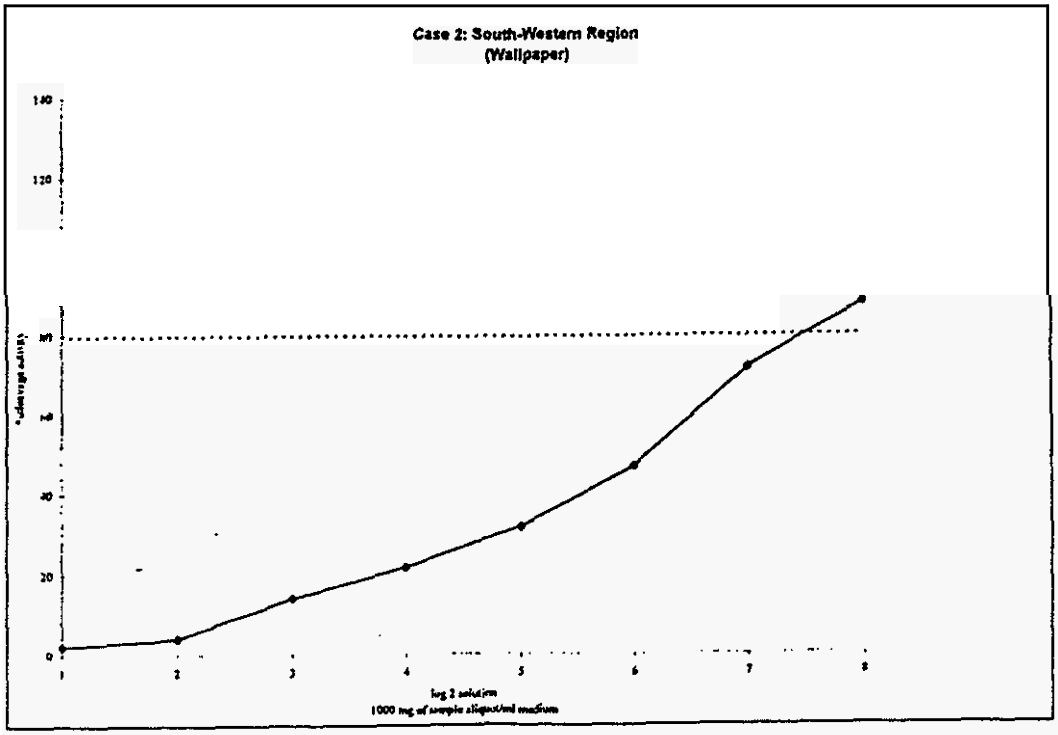
>80% cleavage activity considered non-toxic (normal)

Graph 1

Case 2: (South-Western U.S. region, hot and dry climate):
Paper sheeting of gypsum boards and wooden materials from a kitchen cabinet of a family house were found to be heavily overgrown with black fungal material after a hidden, chronic water leak occurred over many weeks. Several family members (n–8) developed sore throat, nasal irritation, sinus pain, cough, extreme fatigue, skin irritation, chronic headaches, concentration and memory problems over a period of several months. Serum IgE or IgG antibodies to fungi identified in the air and bulk were negligible at the time of testing (several weeks post exposure). Lymphocyte enumeration tests showed very minimal elevations of the total lymphocyte count (CD3) in four of 8 subjects; seven subjects had very low natural killer cell counts (between 3 and 6 %) and markedly elevated mean corpuscular volume (MCV) of the erythrocytes (macrocytosis).

Air and bulk sample analysis:
Culturable fungal airborne concentrations were measured in a range of 459 to 753 CFU/m^3 inside the house compared to the outside with a range of 188 to 288 CFU/m^3. Predominant were Aspergillus (A. *niger*, A, versicolor), *Paecilomyces, Penicillium,* Trichoderma and *S. chartarum*. Bulk sample culture results showed concentrations in the range of 340,000 - 1,120,000 CFU/g (fungi) (*S.* chartarum, A. *niger*, A. versicolor; Aspergillus *spp.*, Trichoderma viride and Penicillium spp.).

The MTT cytotoxicity screening test showed that the bulk sample was highly toxic (up to a dilution step of six to eight) (Fig. 2).



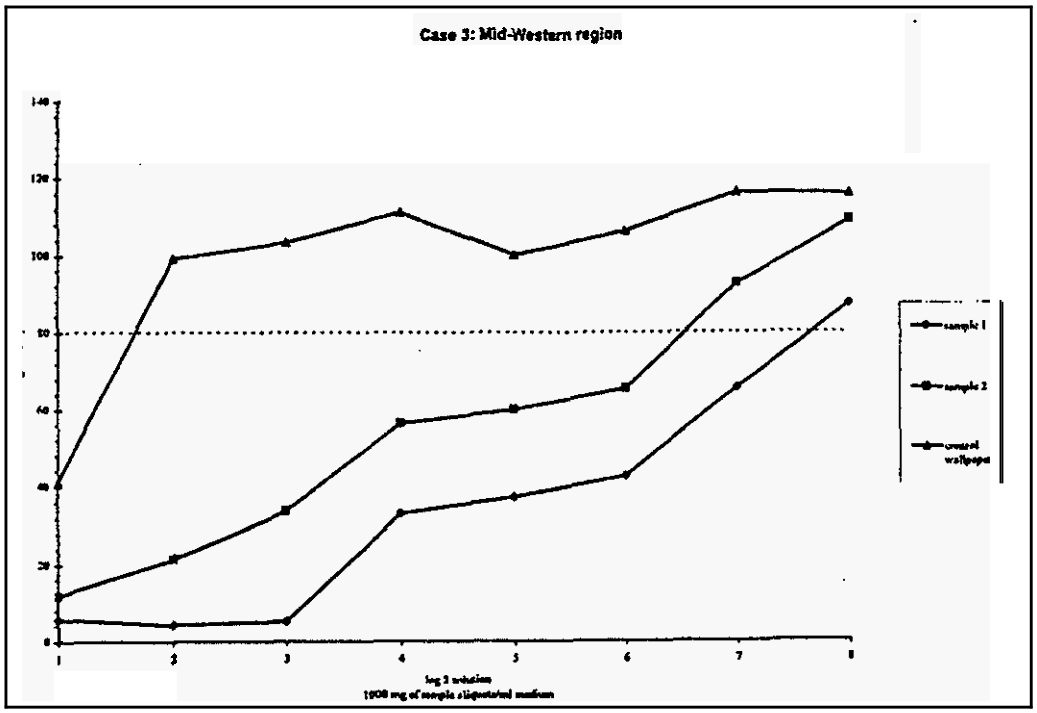
> 80% cleavage activity considered non-toxic (normal)

Graph 2

Case 3 (Mid-Western renion. moderate climate zone. long winter periods):
In the living and food-storage area. next to a central heating and ventilation unit of a multi-unit apartment house, extensive fungal damage was visible on inspected base boards and paper-sheeted gypsum wallboards. Building occupants (n– **12)** complained about a history of irritant skin rashes, asthma and sinus problems, new or worsening allergies. IgG antibodies of ***P. notatum, Aspergillus versicolor*** and ***S. chartarum*** were detected. Natural killer cell (NK) counts were in the lowest (normal) percentile range.

Air and bulk sample analysis:
Indoor airborne fungal concentrations ranged from **269** to **> 2827 CFU/m³**. ***A. versicolor, A. spp., Penicillium, Cladosporium, Chaetomium, Ulocladium*** predominated. Culturable ***S. chartarum*** was detected in three out of **20** air samples. One bulk sample showed a fungal contamination of 4,100,000 **CFU/g,** predominantly ***A. versicolor, A. spp., Penicillium spp., Acremonium*** spp.

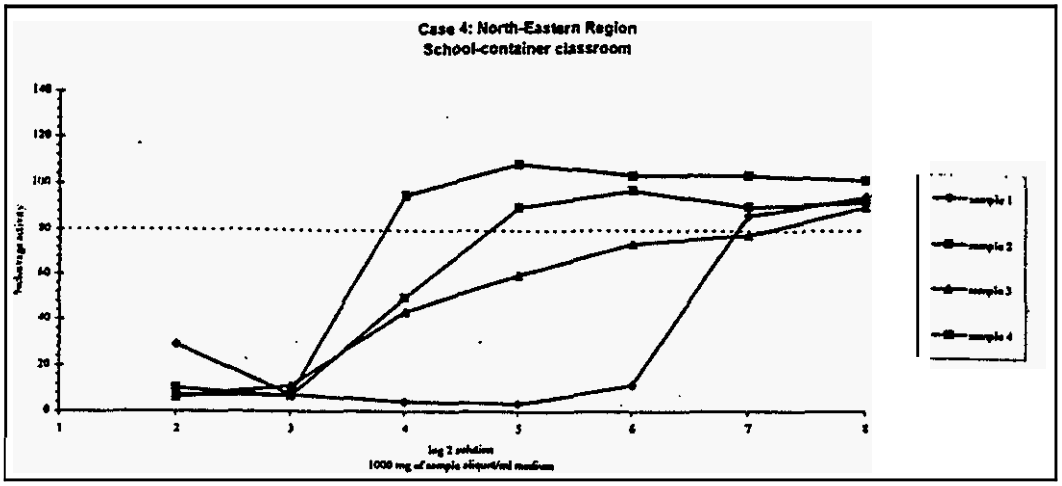
The MTT cytotoxicity screening test of bulk samples showed toxicity between dilution steps two to seven (Fig. 3).



>80% cleavage activity considered non-toxic (normal)

Graph 3

Case 4. (Northeastern U.S. region: cold winters. humid summers. moderate climate)
Visible fungal growth was detected in several areas of a school container structure (a mobile unit) with a long history of roof and window leaks. Personnel (teachers) working several hours a day in this structure complained about severe headaches, lightheadedness, imbalance problems, as well as significant upper and lower airway irritation, asthma and bronchitis. In the three tested subjects, IgG antibodies to ***Trichoderma viride, Alt***



>80% cleavage activity considered non-toxic (normal) Graph 4

ernaria alternata, Cladosporium, Phoma herbarum, *A. fumigatus* and *S.* chartarum **(56** mcg/ml) were above the normal reference range. Natural killer cells (NK) were in the lowest normal percentile range (10-13%). Two subjects were medically removed from the work-site and placed on permanent disability due to the complications **of** their medical problems.

Air and bulk sample analysis:
All cultured bulk samples (wallboard covering, window frame covering, inside wall coverings) were positive for *S.* chartarum (range: 500,000 to 3,500,000 CFU/g) and some of the samples for: A. *versicolor,* Penicillium, Acremonium and Chaetomium.

The MTT cytotoxicity screening test was positive for toxicity up to a dilution level of 3 to 6 (Fig. **4).**

Case **5.** Northeastern region (moderate-coastal climate)
In a wooden-structuresingle-family house black, sooty-looking growth was noticed on various hidden building materials such as wooden **beams,** paper-sheeting of drywall and fiberglass insulation. The occupants' healthcomplaints and findings were related to complications of the eyes, the sinuses and upper airways followed by extreme fatigue, chronic headaches, concentration and memory problems, recurrent lightheadedness and dizziness. Formal, standardized neurobehavioral testing confirmed memory and speech/ verbal dysfunction. The total lymphocyte and the T-lymphocyte count as well **as** the natural killer cells **(NK)** were borderline low. Fungal IgG antibodies were elevated for *Thermoactinomyces* and *Aureobasidium pullulans.* Blood tests were taken several weeks after exposure cessation.

Air and bulk sample analysis:
Indoor air concentrations of culturable fungi ranged from 600 to >4711 CFU/m^3; *S.* chartarum , Penicillium, ***A.*** *niger, Trichoderma, Cladosporium,* and Alternaria were

predominant. Table 1 presents the results of the various bulk samples from different inside locations comparing fungal strain growth characteristics with FFL-cytotoxicity tests and chemical analysis for Satratoxin H and Spirolactones. Table 2 shows the MTT cytotoxicity screening test results of respective samples and comparing it with the Roridin A-EIA results. Agreement between the MTT test and the Roridin A EIA was high; all controls were negative. Results of electron microscopic identification are also listed in table 2. Several of the building materials containing cellulose (fiberglass-paper and gypsum board paper sheeting) showed very high toxicity in both, the FFL and MTT tests. The chemical analysis showed high Satratoxin H or Spirolactones concentrations in various samples. Growth characteristics of S. chartarum under laboratory conditions and cytotoxicity showed notable variation and differences.

Discussion

In this investigation a direct and indirect cytotoxicityscreening method was used to test materials from water damaged buildings for assessment of potentially clinically important toxicity and compared with each other. In addition, S. chartarum derived from these materials was analyzed chemically and by Roridin A-EIA for the presence of macrocyclic trichothecenes. S. chartarum, a known producer of potent mycotoxins (trichothecenes) was identified in all of the investigated problem buildings; in addition a variety of other potentially toxigenic fungi were found in various concentrations (20-22).

Many of the occupants'symptoms reported in our cases could not be explained by allergic pathology, but suggested a toxic health reaction. It is thought that spores or other fungal materials released into the air are responsible for cases of inhalation mycotoxicosis (23). Conventional environmental sampling techniques focus on quantification and speciation of viable or non-viable fungi, but fail to address the toxicity of the materials contributing to indoor air pollution. Toxicity analysis appears necessary to answer certain medical questions and improve our understanding of fungi associated human toxicology. As our results show, the growth characteristics and the presence of S. chartarum are not necessarily indicative of the inherent toxicity of the visible fungal materials.

In the past, easy and rapid toxicity assessment of contaminated building materials has not been possible. Both, the direct MTT test and theindirect FFL test may be. useful and practical screening methods to adequately assess the toxicity for medical purposes under most field conditions. Thecytotoxity screening tests appear sensitiveand reliable for the detection of mycotoxins, in particular of highly toxic satratoxins from *S. chartarum*.

We found different fungal concentrations (primarily of S. chartarum) on original building materials and different concentrations of macrocyclic trichothecenes (i.e., Satratoxin H) or Spirolactone/Spirolactam concentrations in all of the above cases. In thesecases theoccupants complained about significant, although variant, health problems (upper and lower airway, skin, eye, central nervous system dysfunction) associated with massive indoor moisture and fungal problems. The differences may be related to the specifics of the exposure, the duration and chemical composition. Other toxic fungal metabolites may have caused a cytotoxic reaction in either the MTT or the FFL test, although the chemical analysis and the Roridin A-EIA indicated a high concentration

Table 1: Characteristics of *Stachybotrys chartarum* strains isolated from building samples (Case 5).

Sample Number / Location:	Fungal strain growth of Stachybotrys chartarum:	Cytotoxicity* FFL-test	Chemical Satratoxin H	Concentration*: Spirolactone/lactams
# 2-4 / Kids room	compact	high	low	high
		high	low	high
# 6 / fireplace room	compact	minimal	ND	low
	moderate	moderate	ND	low
#10 / office / dry wall paper	compact	high	high	high
fiberglass	moderate	low	low	high
office dry wall paper	compact	high	high	moderate
	moderate	low	low	moderate
basement ceiling tile	compact	moderate	low	moderate
	moderate	low	low	moderate
	compact	low	ND	low
	fast	low	low	high

control samples: all negative in all assays
• of isolated strains

Table 2: Results of mycological investigation, MTT-cytotoxity, EIA-analyses of field samples.

Lab. No	Sample location	Visible growth	ELMI	Fungal strain	MTT cytotoxity	EIA (Roridin A)*
1	Control	-	PNC	ASP, PNC	-	0
2	Fg/Ceiling kids room	+++	PNC, S.c.	PNC	+	1,6-4,6
3	Fg/Ceiling kids room	+++	S.c.	Periconia spp.	-	0.8
4	Fg/Ceiling kids room	+++	S.c.	S.c., PNC spp.	+	1.4
5	Wall paper/fireplace	+	S.c.	Alternaria, A. niger	+++	27.6
6	Fg/wall paper/fireplace	+	P. expansum	P. expansum	++	21
7	Control / fireplace	-	-	-	-	-
8	Control / kids room	-	-	yeast	-	-
9	dry wall paper / office	+	S.c.	A. niger, S.c.	+	7.3
10	Fg/drywall paper/ office	+++	S.c.	A. spp., S.c.	++++	282.5
11	basement ceiling tile	++	S.c.	S.c., A. spp.	++	31.3

ELMI = Electronmicroscopy of original sample
PNC = Penicillium, ASP = Aspergillus, S.c. = Stachybotrys chartarum
*Enzyme immunoassay: ng Roridin A equ./ml sample (from crude extracts used for MTT test)

and presence of macrocyclic trichothecenes. The detected Spirolactones/lactams have been discussed as possible immunological complement inhibitor. However, clinical experience currently appears insufficient *to* comment on their biologic relevance in humans.

Overall the agreement between the *tests* was good and differences may be related *to* specific local and sample variations (paper sheeting from gypsum board, fiberglass insulation paper backing, wood, and other high cellulose containing materials). Further, differences between the **test-results** for materials from the same origin (see tables) may be related *to* methodological differences between the direct (MTT or Roridin A-EIA) and indirect (FFL and chemistry analysis) cytotoxicity testing. Fungal cultures for the FFL *test* were obtained from the material and then grown under "ideal" laboratory conditions (FFL-method), which are not necessarily representative of "in-vivo" conditions (see different climate zones of sampling sites). **Also,** different nutrient properties of the building materials (substrate) may influence the differences in the test results. The water concentration; the cellulose content and possibly the chemical treatment of the tested building materials may have influenced the performance and metabolism of the mycotoxin producers.

The FFL test has been reported *to* be very sensitive to trichothecene, but requires that the technician performs an interpretation and (subjective) rating of a microscopic examination of the cell line. The MTT cytotoxicity test with the swine-kidney cell line is a robust and sensitive colorimetric test method, which has been used in many other applications such as food-safety tests and environmental screening (24). Concentrations of trichothecene mycotoxins of 0.01 µg/ml (Verrucarin) to T-2 Toxin (0.4 µg/ml) Roridin A (<0.1 µg/ml), Satratoxin H(1.6 µg/ml) and nivalenol(l2.5 µg/ml) have been measured with the SK cells. Immunochemical methods **(EIA)** such as the Roridin A ELISA *test* may be useful in the future as a simple direct test preparation, requiring minimal preparation and time, and with a very high sensitivity (up *to* picogram concentrations) (25). The Roridin A-assay cross-reacts with Satratoxin H and may be used as an indicator, In general, non-contaminated, similar building materials should be used as internal control samples if available, since all cell lines may also be affected by other non-fungal toxins stemming from the various construction materials. Otherwise, confirmatory *tests* may be necessary to supplement the MTT or FFL *test* results.

Some authors argue that several analytical techniques should be used *to* investigate building-related health hazards. A novel in-vitro toxicity test that utilized boar spermatozoa was recently reported *to* be useful for detecting toxins of microbial origin toward eukaryotic cells not detectable in moisture-damaged building materials (with dominant colonizers of ***S. chartarum, Penicillium and Aspergillus spp.).*** (26).

In addition *to* mycotoxins, bacterial endotoxins **(27, 28)** ß-D-Glucans **(29)** and ergosterol as a nonspecific measure for fungal exposure (30) have been discussed as possible environmental exposure markers. However, clinical experience is still limited utilizing such markers in practical settings.

In summary, the cytotoxicity tests, such as the direct MTT-test, appear to be a reliable quickscreening method. It may be beneficial in health-related building investigations in addition to conventional mycological sampling and identification methods.

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Review article

Effects of damp and mould in the home on respiratory health: a review of the literature

Peat JK, Dickerson J, Li J. Effects of damp and mould in the home on respiratory health: a review of the literature.
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This review examines whether there is a direct or indirect relation between damp or mould in the home and respiratory health. Home dampness is thought to have health consequences because it has the potential to increase the proliferation of house-dust mites and moulds, both of which are allergenic The results from the many studies conducted to investigate whether damp and mould are associated with health outcomes are difficult to compare because the methods of measuring exposures and health outcomes have not been standardized. However, the studies that have been conducted in children are probably the most reliable because the confounding effects of active smoking or occupational exposures are absent, and because the presence of symptoms of cough and wheeze have been consistently investigated in many studies The increased risk of children having these symptoms if the home has damp or mould is fairly small with an odds ratio that is generally in the range 1.5–3.5, these estimates being statistically significant when the sample size has k e n large enough. This range is consistent with the measured effects of other environmental exposures which are considered important to health, such as environmental tobacco smoke or outdoor air pollutants. The potential benefits of reducing mould in the home have not been investigated, and the few studies that have investigated health improvements as a result of increasing ventilation or reducing damp in order to reduce house-dust mite levels suggest that this intervention is expensive. requires a large commitment. and is unlikely to be successful in the long term. This implies that houses need to be specifically designed for primary prevention of respiratory problems associated with indoor allergen proliferation rather than using post hoc procedures to improve indoor climate and reduce allergen load as a secondary or tertiary preventive strategy.

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Damp in the home may have health consequences because of its action in increasing the proliferation of housedust mites and moulds both of which are allergenic. However, it is difficult to collect direct evidence that building design influences the indoor environment and, in turn. allergen levels and respiratory health. In practice, it is not possible to monitor closely indoor microclimates, which are complex and are influenced by many factors, including building materials ventilation, water content, and the effects of heating and cooling; therefore, the factors which influence exposure to indoor allergens have not been accurately identified. Moreover, there are inherent limitations in the design of the studies which can be used to investigate whether there is a relation between indoor exposure and clinical outcome. Because cohort studies are constrained by the financial and practical problems of measuring exposure over extended periods the studies from which there is evidence have been cross-sectional or case-control in design, because these provide more immediate results but there are obvious problems in using their results to infer direct causal effects

This review summarizes the literature which has investigated respiratory health outcomes in rela-

Table 1. Associations between house-dust mite levels and housing characteristics that have been reported

Authors	Study design	Effect
Cabrera et al. (1995) (64)	Der p 1 levels in beds of asthmatic patients in 10 homes in Spain	Der p 1 levels were 4–8-fold lower in homes where dehumidifier was used
Chan-Yeung et al. (1995) (13)	Der p 1 levels in beds and floors of asthmatics in 57 homes in Vancouver and 63 in Winnipeg	Der p 1 levels related to age of home, storey, type of heating, and number of occupants
Colloff (1992) (58)	House-dust mite numbers in beds of 23 people with atopic dermatitis in UK	House-dust mite numbers slightly higher in mattresses in damp homes
Dornelas de Andrade et al. (1995) (65)	Der p 1 levels from cross-section of 108 homes in France	Der p 1 levels lower in homes with low relative humidity but no relation to presence of mechanical ventilation, water vapour, heating, or air vents or to use of humidifiers
Dotterud et al. (1995) (66)	House-dust mite numbers in beds of 19 atopic and 19 nonatopic children in Norway	House-dust mite numbers slightly higher in homes with poor ventilation, increased humidity, and water leaks
Fletcher et al (1996) (23)	Der p 1 levels in beds and floors of nine homes with mechanical ventilation heat recovery units and nine control homes in UK	Der p 1 levels not significantly lower in homes with mechanical ventilation heat recovery units
Harving et al. (1993) (19)	House-dust mite numbers in 96 homes of asthmatics in Denmark	House-dust mite numbers related to indoor humidity and air-exchange rates
Kalra et al. (1992) (16)	Der p 1 levels in beds and floors of homes of 40 asthmatic patients in UK	No correlation between Der p 1 levels and house type, ventilation, or presence of double glazing
Kuehr et al. (1994) (67)	Der p 1 levels in beds of cohort of 1291 children in Germany	Der p 1 levels higher in rooms with damp spots, higher relative humidity, and low storey level, and lower in homes with underfloor heating
Lintner & Brame (1993) (11)	Der p 1 levels in beds or carpets in 424 homes in USA	Der p 1 levels lower in homes with air-conditioning, especially in summer months
Malainual et al. (1995) (68)	Der p 1 levels in beds in 630 buildings in Thailand	Der p 1 levels not different between regions or building types (single house, shop, slum house, and hotels)
Munir et al. (1995) (69)	Der p 1 levels in beds and floors of 130 homes of asthmatic children in three regions of Sweden	Der p 1 levels higher in homes with dampness problems and in homes without basement; also higher in homes with higher relative humidity and poor ventilation
Plácido et al. (1996) (70)	Der p 1 levels in floors of 59 asthmatics, 36 house-dust-mite-sensitized subjects, and 23 controls in Portugal	Higher Der p 1 levels associated with older homes, signs of dampness, and presence of carpets
Sundell et al. (1995) (71)	Der p 1 levels in beds and floors from 30 homes with levels above 2 mg/ml and 30 homes with levels below 1 mg/ml in Stockholm	High Der p 1 levels associated with high indoor compared to outdoor humidity and with low air-exchange rates in home
Tovey et al. (1996) (72)	Der p 1 levels in beds and floors of over 600 randomly selected children in nine regions of Australia	No difference in Der p 1 levels between storey, wall construction, age of home, carpetting, presence of mould, or ventilation type
van Strien et al. (1994) (20)	Der p 1 levels from 516 houses in The Netherlands; relative humidity measured in 19 homes	Der p 1 levels higher in homes with observed signs of dampness (total sample) and higher absolute humidity (subsample)
van Strien et al. (1995) (73)	Der p 1 levels from beds of 46 "high-risk" infants (with atopic mothers) and 58 "low risk" infants in The Netherlands	Der p 1 levels lower in beds of infants with new mattresses or blankets and no plastic sheeting over mattresses
Wickman et al. (1993) (74)	House-dust mite numbers in dust from 65 homes of house-dust-mite-sensitized children in Sweden	House-dust mite numbers higher in mattresses in basement and ground floor bedrooms and in tight or damp homes
Wickman et al. (1994) (75)	Der p 1 levels in mattresses from 70 houses in Sweden	Der p 1 levels lower in homes with natural ventilation and presence of basement
Zock et al. (1994) (76)	Der p 1 levels from homes of 228 asthmatic children in The Netherlands	Der p 1 levels lower in homes with carpet compared to hard floors

tion to housing characteristics or the presence of damp or mould in the home. All of the papers from the last 15 years which could be accessed via MEDLINE are included.

Housing characteristics associated with house-dust mite proliferation

Indoor humidity is thought to have a major controlling influence on house-dust mite populations. In clinical terms, this is important because house-dust mite exposure is one of the best documented environmental causes of asthma (1–4). Because very low house-dust mite allergen (Der p 1) exposure levels have been associated with sensitization and symptoms (5), no safe threshold of exposure to Der p 1 has been confirmed.

Levels of Der p 1 are almost certainly influenced by local conditions because much higher levels are found in humid regions (3, 6–8) and because levels

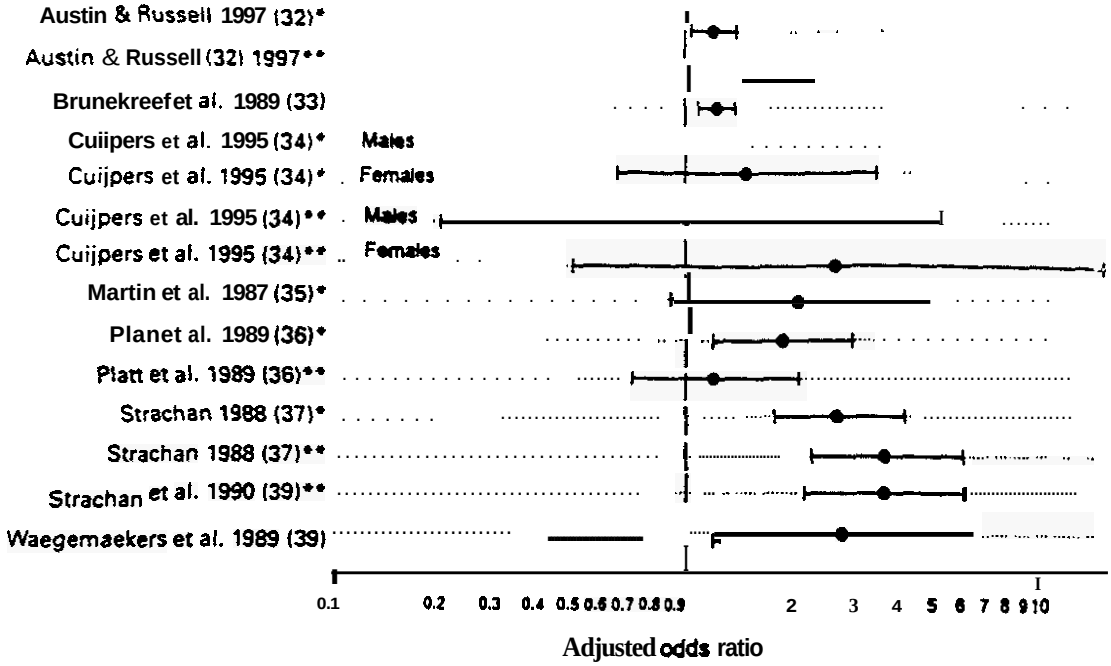

Fig. 1. Reported associations between wheeze in children and damp or mould in home. Data shown are odds ratios and their 95% confidence interval. Associations with damp are indicated by • and with mould by **. In studies where both mould and damp have been combined, no annotation is shown.

undergo spontaneous seasonal changes (9–15), although such changes have sometimes been small (16) and have not always been demonstrated (17). The vast range of Der p 1 levels in homes within the same climate (3, 8, 13, 18) also supports the hypothesis that indoor microclimate has a large influence. A relationship between measurements of indoor relative humidity and Der p 1 levels in homes has been established in some studies (8.16, 19, 20), but not others (13).

The literature which has associated Der p 1 levels with specific home characteristics has been reviewed (21). Table 1 shows that housing characteristics which can be assumed to increase indoor humidity have been associated with higher house-dust mite numbers or with higher allergen levels in some, but not all. studies It has been suggested that mechanical ventilation may have a major effect on indoor climate in cold regions but is unlikely to be effective in regions which have a humid outdoor climate for a significant part of the year (22, 23). The finding that beds have higher Der p 1 levels than floors (12, 24) and that bed, but not floor, levels are associated with the severity of airway hyperresponsiveness (3) suggests that the microclimate that influences allergen exposure from beds may be more relevant to respiratory health than that which influences exposure from floors.

There is some evidence that post hoc modifications to the home environment can reduce allergen levels In the USA, the use of air-conditioning to modify indoor climates reduced house-dust mite populations and subsequent allergen production (11), and, in Denmark, modest reductions in house-dust mite counts were monitored after increased indoor air-exchange rates to reduce humidity (19, 25), but the counts were low at the onset of the trial, and the changes achieved were too small to have clinical importance. In the UK, acaracide treatment of beds and floors has been shown to reduce allergen exposure significantly (26) and to improve airway responsiveness marginally in housedust-mite-sensitized asthmatic children (27).

Association between indoor damp or mould growth and health

In the USA, exposure to *Alternaria* from outdoor sources has been associated with asthma exacerbations (28), and, in Australia, we found an important association between allergic sensitization to *Alternaria* and childhood asthma in inland regions where *Alternaria* levels are thought to be high as a result of farming practices and/or climatic conditions (29). Although children in Australia and New Zealand become sensitized to other moulds such as *Cladosporium*, *Penicillium*, and *Aspergillus*, this does not appear to have clinical importance in terms of being a risk factor for the development of asthma (30.31). This suggests that levels of expo-

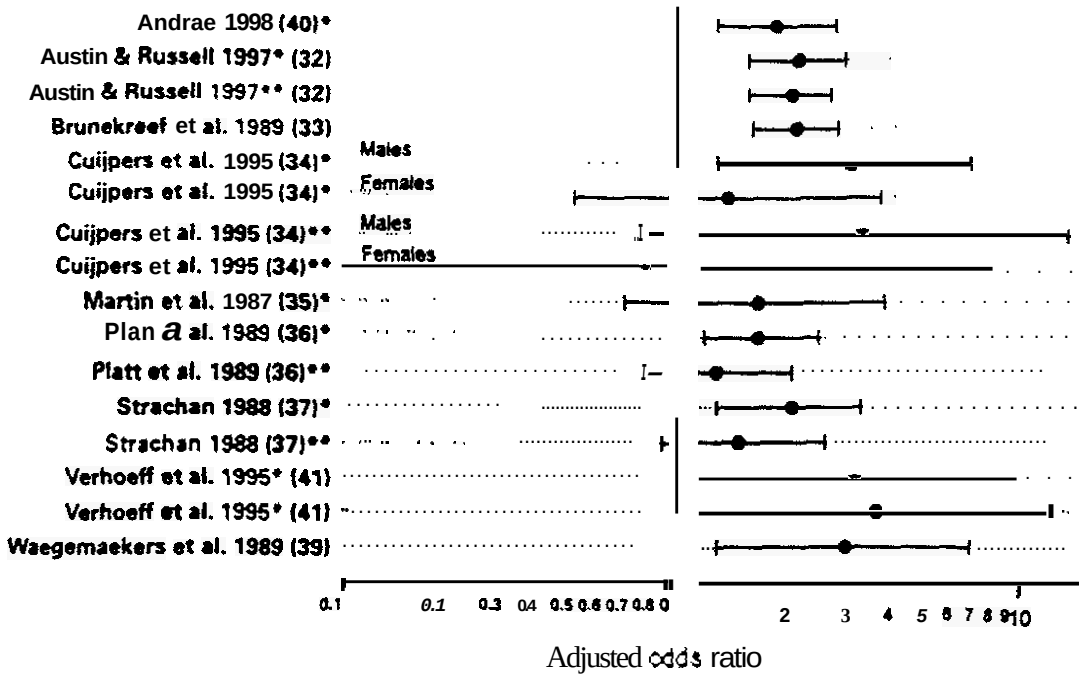

Fig. 2. Association between cough in children and damp or mould in home Data shown are odds ratios and their 95% confidence interval. Associations with damp arc indicated by * and with mould by **. In studies where both mould and damp have been combined. no annotation is shown.

sure to these moulds may be above the threshold needed to cause sensitization but below that which promotes ainvay inflammation, or that these particular moulds are less potent than *Alternaria* in causing ainvay abnormalities.

The two symptoms which have been investigated most often in relation to the home environment are cough and wheeze. Fig. 1 shows the odds ratios &om the studies which have investigated an association between damp or mould in the home and wheeze in children (32–39), and Fig. 2 shows the odds ratios for cough (32–41). One study which did not find a significant effect between damp and respiratory symptoms but which yielded insufficient information to compute an odds ratio (42) is excluded.

Fig. 3 shows the odds ratios for cough and wheeze in adults associated with damp and mould in the home (36, 43–45), and Fig. 4 shows the odds ratios for asthma in both children and adults (33, 39–41, 43–50). Approximately half of the studies show a significant association between respiratory symptoms and the presence of damp and mould. The odds ratios from almost all studies are greater than unity, although the estimates from studies with a small sample size are not statistically significant. The only study which has examined the relation between the seventy of asthma and the amount of damp in the home was a case-control study of asthmatic and nonasthmatic subjects in which a modest correlation between a damp score and an asthma seventy score was found (50).

Housing characteristics associated with mould growth

There are no practical and accurate methods with which to monitor mould exposure. Objective methods such as air sampling, weight gain of wood blocks, or cultivation of spores from dust have been tested (36, 38, 49, 51–54), but only a modest agreement between objective sampling methods and questionnaires has been found (39, 49, 50). Most epidemiologic studies have relied on questionnaires completed by occupants to measure the presence of damp or mould. but evidence from two studies suggests that occupants are more likely to report their homes as having damp or mould than are research investigators, with as much as 30% disagreement (36, 41). and a recent study suggests the opposite – that is, that occupants are Less likely to report homes as being damp (50).

Only a small number of studies have investigated the indoor factors that increase mould levels or the influence of outdoor mould levels on indoor levels As with house-dust mite levels, the wide variations in mould levels between homes (38) suggests that indoor microclimate is also an important determinant of mould exposure. In Sweden, moulds present in winter were associated with housing

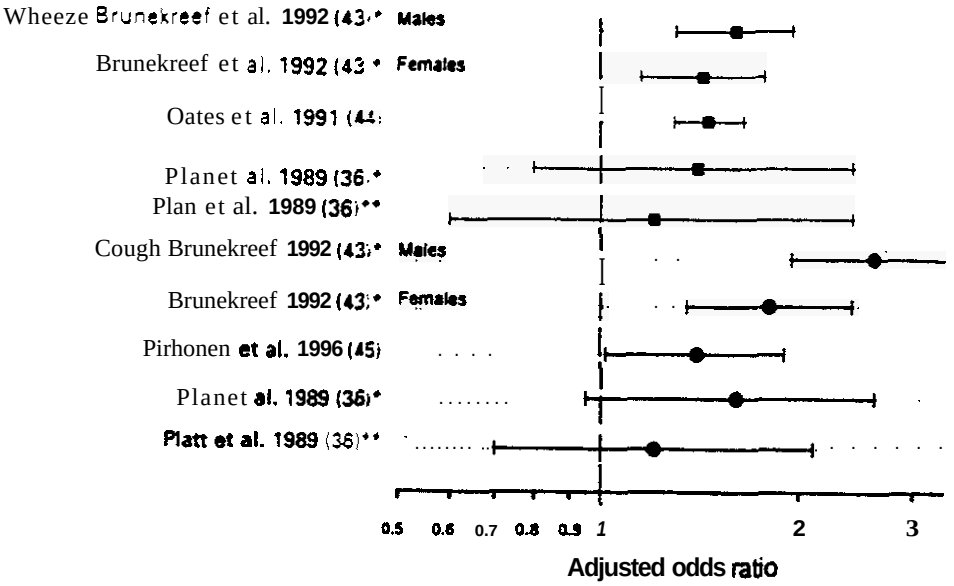

Fig. 3. Association between cough and wheeze in adults and damp **or** mould in home. Data shown are **odds** ratios and their ***95%*** confidence interval. Associations with **damp** are indicated by • and with mould by **. In studies where both mould and damp have been combined, **no** annotation is shown.

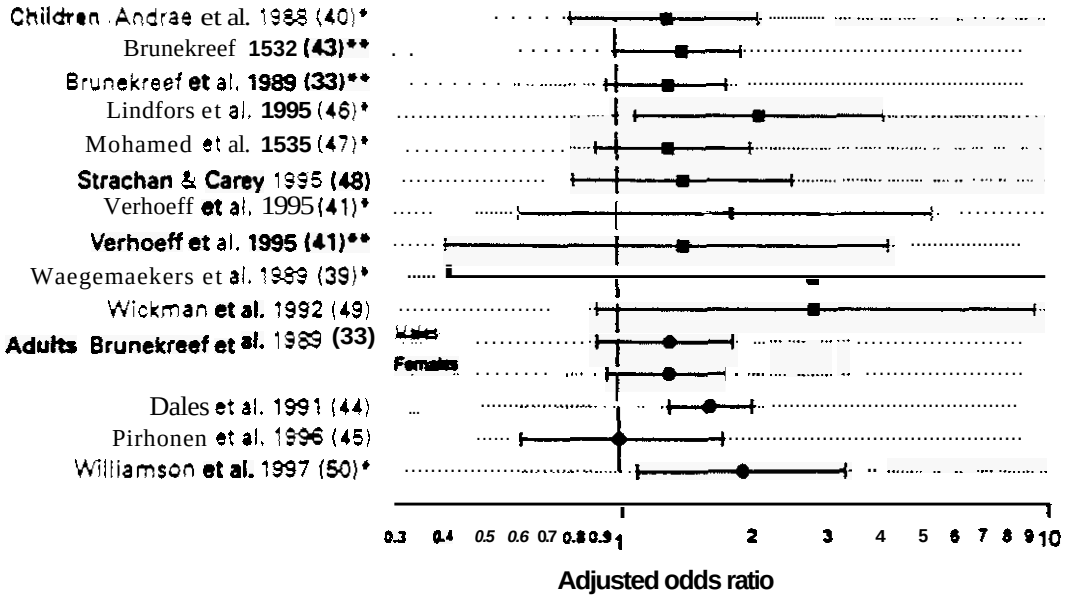

Fig. 4. Association between **asthma** in **children** and adults and damp **or** mould in home. Data shown are odds ratios and **their** 95% confidence interval. Associations with damp are indicated by * and with mould by **. In studies where both mould and damp have **k e n** combined. **no** annotation is shown.

characteristics (*Alternaria* with thermal insulation, *Aspergillus* with homes rather than units and with wall-to-wall carpets, and *Cladosporium* **with con-**crete slab ground **floors),** and there was a marginal association between mould levels and **length** of time spent showering or visible condensation on window panes **(49).** although only a small number of homes were studied, **so** that the findings may not be generalizable. In The Netherlands. indoor mould levels were found to be only weakly related to outdoor levels. to damp in the home, or to home characteristics such as relative indoor humidity **(54. 55).** and. in San Francisco, outdoor mould levels were higher than indoor levels in new occupancy units (53), and the indoor and outdoor species were very different (54, 56).

Few studies have investigated the efficacy of interventions to reduce mould growth. In Denmark. a small. uncontrolled study of 23 homes showed that increased ventilation and reduced humidity significantly reduced indoor mould levels (56). and. in the UK. air infiltration units reduced levels of airborne moulds but had no effect on airborne Der p 1 levels (57).

Methodological considerations

It has been difficult to quantify the relation between housing design and health outcomes because of the limited study designs which can be used. Randomized controlled trials cannot be used in this context, and studies of birth cohorts are constrained by the financial and practical problems of measuring long-term exposure. As a result, the available evidence comes from case-control or cross-sectional studies, because they provide more immediate results, but causation cannot be inferred From such studies, a small and statistically nonsignificant effect would be expected if the real effect is small and therefore difficult to quantify, if only a minority of the sample are susceptible to the effects of exposure, or if there is misclassification bias in either the outcome or the exposure variables On the other hand, the effect will be overestimated in studies in which the subjects associate home dampness with symptom outcomes; for example, the study which was undertaken in response to concerns about damp (39). or in which subjects living in dry or nonmouldy homes have a less accurate recall of symptoms Such misclassification bias may explain why symptoms have been associated with visual signs of dampness or mould growth but not with air spore counts (36), and with mould growth but not with an objective test of exercise-induced lability (37).

More certainty can be attributed to findings from studies of children because the confounding effects of cigarette smoking and occupational exposures are absent. It is also important to consider that some groups, such as subjects with specific allergies, may be at greater risk of having symptoms if they live in a home with damp or mould present. Thus, it is important to test the hypothesis that sensitized subjects experience more respiratory problems if their home presents high levels of exposure to the allergen to which they are sensitized. Because none of the reported studies have adopted this approach, the effects of damp or mould in the home on susceptible subjects may have been underestimated. This notion of susceptibility is supported by the few studies which have demonstrated larger effects of damp or mould housing in atopic subjects (41, 49, 58).

In the four studies which have presented unadjusted and adjusted odds ratios (39. 41. 43, 44). the estimates are very similar, suggesting that there are no known factors which may have a confounding influence on the effects Although it has been suggested that the effects of exposure to damp and to other factors such as environmental tobacco smoke are independent (40), no studies have investigated possible interacting' effects of damp or mould with other documented risk factors.

It is also possible that "surrogate" effects may be present when assessing possible roles of environmental exposures Studies which show that children living in homes with coal or wood heating have less allergic illness (59) or that symptoms change when children move house (60) suggest that housing design may affect health, although other lifestyle factors which have an inherent relationship with changed lifestyle, such as diet, types of furnishings, and hygiene, could make a major contribution to the effect.

Summary

Clearly, most people are exposed to house-dust mites and to a wide range of mould spores in their home, but whether certain home characteristics lead to increased allergen exposure and, as a consequence, to respiratory health effects remains unclear. The symptoms for which there is most evidence. because they have been monitored most consistently, are wheeze and chronic cough. The increased risk of having these symptoms if the home is damp or mouldy is fairly small, with an odds ratio that is generally in the range 1.5–3.5, and may be statistically significant depending on sample size. This range is consistent with the measured effects of other exposures which are considered important such as environmental tobacco smoke or outdoor air pollution.

It is difficult to judge whether housing design. per se, leads to health problems. It is thought that the major sources of *Alternaria* are outdoors whereas the sources of *Aspergillus* and *Cladosporium* are typically indoors. However, indoor exposures are likely to be a mixture of spores from indoor and outdoor sources and are likely to present more constant exposure levels than outdoor levels, which undergo large, intermittent fluctuations Thus, tight or poorly ventilated homes may increase exposure to indoor allergens. whereas well-ventilated homes may increase intermittent exposures to outdoor moulds

Future directions

In the future, studies with large sample sizes will be needed to measure whether intermittent peak exposures or low cumulative exposures to indoor

allergens pose a clinically important risk. The design of such studies will need to take account of problems such as variability in assessing exposures and notions of individual susceptibility. It is also important to determine whether living in a home with high house-dust mite or mould exposure as a result of dampness or poor ventilation increases the severity of existing airway disease or has a more profound effect of initiating respiratory illness in previously disease-free subjects

Despite the problems in demonstrating a cause-and-effect relation between environmental exposure and health effects, it is essential to initiate strategies to prevent respiratory disease, because symptoms of asthma, wheezing. and cough impose a high social burden and cost on society, especially for children (61-63). Because people spend most of their time indoors, it is important that the home environment does not contribute to poor health. Although standards for outdoor air quality and for indoor air quality in public buildings are now in place, no standards have yet been developed to maintain air quality in the home. In practice, it may be more cost-effective to design houses that prevent indoor allergen proliferation and the onset of related illnesses rather than to invest in expensive post hoc procedures to improve homes with established allergen colonies The collective evidence is beginning to suggest that homes which are specifically designed to minimize indoor humidity may make an important contribution to the respiratory health of both children and adults who have an allergic disposition.

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Correlation between the prevalence of certain fungi and sick building syndrome

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Abstract

Objective—To examine the role of fungi in the production of sick building syndrome.

Methods—A 22 month study in the United States of 48 schools (in which there had been concerns abour health and indoor air quality (IAQ)). Building indoor air and surface sampla, as well as outdoor air samples were taken at all sires to look for the praencc of fungi or their viable propagules.

Results—Five fungal genera were consistently found in the outdoor air and comprised over 95% of the ourdoor fungi. These genera were *Cladosporium* (81.5%), *Penicillium* (5.2%), *Chrysasporium* (4.9%), *Alternaria* (2.8%), and *Aspergillus* (1.1%). At 20 schools, there were significantly more colony forming units per cubic metre (CFU/m³) ($p<0.0001$) of propagules of *Penicillium* species in the air sampla from complaint areas when compared with the ourdoor air samples and the indoor air samples from non-complaint areas. At five schools, there were more, although not significant ($p=0.10$), *Penicillium* propagules in the air sampla Erom complaint areas when compared with the ourdoor air samples and the indoor air samples from non-complaint areas. In 11 schools, the indoor air (complaint areas) fungal ratios were similar to that in the ourdoor air. In these 11 schools *Stachybotrys atra* was isolated from swab samples of visible growth under wetted carpets, on wetted walls, or behind vinyl wall covering. In the remaining 11 schools, the fund ratios and CFU/m³ of air were not significantly different in different areas. Many of the schools rook remedial action that resulted in an indoor air fungal profile chat was similarto chat outdoors.

Conclusions—Propagules of *Penicillium* and *Stachybotrys* species may be associated with rick building syndrome.

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Keywords: fungi; rick building syndrome; indoor air quality; spores

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Reports abour buildings wirh air related problems have appeared increasingly often after the early 1970s, although this problem has certainly been with humans for centuries.[1,2] Sick building syndrome (SBS), a commonly used term for symptoms resulting from problems with indoor air quality (IAQ), was first people in certain buildings in 1982. The first official study of SBS that examined more than one structure was published in 1984.[3] Sick building syndrome has been difficult to define and no Single cause of this malady has been identified.[4]

Complaints common to SBS include allergic rhinitis, difficulty in breathing, headaches, flu-like symptoms, and watering of the Numerous studies have been carried out in an attempt to elucidate the cause of SBS.[5] Early srudies showed char many of the reported causes of SBS were undesirably high levels of known respiratory irritants such as nitrogen and sulphur dioxides, hydrocarbons, and particulates,[6] known or suspected carcinogens such as asbestos, radon, formaldehyde, and tobacco smoke,[7] or chemicals being released by new building materials.

Although fungal spores are universal atmospheric components both indoors and outdoors and arc now generally recognised as important causes of respiratory allergies,[8–10] there are few studies showing which fungi and spores are associated with IAQ problems.[11] This scudy was made possible due to our association with an IAQ company. The uniqueness of this study was that the sires were made available because the school officialscontacted the IAQ company. This allowed us access to all the samples, data, questionnaires, and occupant generated complaints from schools that were experiencing IAQ problems.

Although no one cause for the symptoms induced by IAQ problems is likely to exist, the presence of fungi in sick buildings is becoming consistently associated with this problem.[12–17] Fungal contaminadon in indoor environments has been shown to produce allergies in occupants of these buildings.[18–20] Even though the IAQ company's investigations were broadly based and in depth, the presence of fungi was the primary focus of this study, in which we present evidence for the role of *Penicillium* species and *Stachybotrys* species in buildings with L4Q problems.

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Materials and methods

SURVEY PROCEDURES

The 22 month scudy examined 48 schools chat were experiencing L4Q problems. There were in states along the United States Gulf of Mexico and the Atlantic seaboard. The sires were surveyed with the following criteria: collection of building characterisadon data based on direct inspecdon and interviews with building occupants; building characterisadon including measurement of temperature and

and air conditioning (WAC) systems and physical examination of the building; particulates, CO, and chemical measurements; inspection of sites that had been wetted or showed mould growth; swab samples and air samples taken by an Andenen air sampler; and administration of a questionnaire to building occupants, or access to occupant generated complaints (letters to school officials) and other complaint surveys conducted by other agencies—such as public health departments.

The questionnaire chat was administered by the IAQ company was designed to determine the areas or rooms in which the occupants had complaints about the IAQ. The questionnaire asked for such information as the nature of the complaint, symptom patterns, timing patterns, and observations about building conditions chat might explain the symptoms found The questionnaire's answers and comments, dong with the occupant generated complains and surveys Lorn other agencies, were placed into the following categories: (a) type of symptoms; (b) when do the symptom start; (c) when do the symptom go away; (d) when an the symptoms wont; (e); pre-existing symptom (allergies, asthma, etc); (f) discomfort complaints (noise, temperature, odours, etc); and (g) complaint areas (rooms).

Non-complaint areas were also identified. The minimum criteria for designation as a non-complaint area were (a) all occupants, whose primary location was the non-complaint area, had no IAQ complaints, and (b) the HVAC system was separate Lorn any complaint areas.

MICROBIOLOGICAL IDENTIFICATION

Air samples wen taken with a two stage Bioaerosol Sampler (Model 2000 Andersen Samplers, Atlanta, GA, USA) at a calibrated flow rate of 28.4 l/min for 5 minutes. During sampling, the Andenen samplers were placed about 1 m above floor level. Sabouraud's Dextrose agar (SDA) pH 5.6 was used for air sampling and swab sampling. Plates were incubaud at 22°C and 90% relative humidity (RH) for up to 14 dam. The isolated fungi were identified with. standard identification techniques."' Colony forming units per cubic metre (CFU/m') of air were calculated with the formula:

CFU/m' = (number of CFU / ((number of minutes sampled)(1 ft'/minute))) (35.3 ft'/m')

The total fungal CFU/m' for each air sample was calculated and the ratio for each organism per sample was determined. The results were entered according to the area chat was sampled (the outdoor air sample areas. the indoor air samples complaint areas or the indoor air sample non-complaint areas) and the average CFU/m' and ratio, in terms of percentage, for each organism was determined for each area examined.

Using sterile swabs, samples were taken from areas of visible fungal growth, HVAC systems, wetted areas, standing water, dead air spaces, and areas of dust accumulation. The swabs transport to the laboratory or streaked undiluted on to agar pH 5.6 plates. At the laboratory, the swab up was placed into a sterile tube containing 10 ml Sterile phosphate bufferedsaline and vigorously vortexed for one minute. Samples (100 µl) were pipetted on to agar plates (pH 5.6) and spread with sterile rods. The plates were incubated at 22°C and 90% relative humidity for up to 14 days. The fungi were identified and the fungal growth on the plates was estimated with the following titeria: 0 CFU, no growth; 1–5 CFU, very light growth; 6–10 CFU, light growth; 11–30 CFU, medium growth; 31–50 CFU, heavy growth; and >50 CFU, very heavy growth.

CARSON DIOXIDE MEASUREMENTS

The CO_2 content of the air, expressed in parts per million (ppm), was monitored with a Ventostat CO_2 Sensor (Model 107011071, Telaire System. Delspo, Sweden).

CHEMICAL MEASUREMENTS

An independent laboratory was contracted by the IAQ company to perform sampling for various indoor air components such as formaldehyde, nitrogen dioxide, hydrogen sulphide, sulphur dioxide, and carbon monoxide. Formaldehyde samples were collected wirh a Sensidyne/Gastec (Sensidyne/Gastec, Goleta, CA) collector in association with colorimetric indicator tubes with a lower detection limit of 0.1 ppm. Nitrogen dioxide, hydrogen sulphide, sulphur dioxide, and carbon monoxide samples were collected with a MultiRAE PGM-50 gas sampling device (Rae Systems, Sunnyvale, CA, USA). The collector was calibrated before sampling for each particular component. Outdoor and indoor samples were taken in the complaint and non-complaint areas. All Sampling was performed in accordance to the manufacturers suggested use.

MEASUREMENTS OF PARTICULATES, TEMPERATURE, AND RELATIVE HUMIDITY

Airborne particles were counted with an APC-1000 Airborne Particle Counter (Biotest Diagnostics, Denville, NJ, USA). The APC-1000 detects particles relative to four thresholds: >0.3, 0.5, 1.0, and 5.0 µm. The APC-1000 also measures temperature and relative humidity.

REMEDIAL ACTION IN BUILDINGS

Any building materials that showed physical deterioration were removed and replaced. Existing microbial contamination on intact and structurally sound surfaces was cleaned and treated with an approved disinfectant. The remedial measures to the HVAC consisted of the removal of visible surface contaminants and the cleaning of air side surfaces of all internal air handling surfaces including, but not limited to, fans, coils, drain pans, filter racks, motors, dampers, and specific air ducts. Any damaged or delaminated insulation within the airducts being cleaned and sanitised was replaced. All work areas inside the air handlers, air duns, and equipment rooms were isolated and kept under negative air pressure with high efficiency particulate arrestor filtered negative

Incidences per 100 employees (95% CI) of reported complaints and symptoms regarding indoor air quality (IAQ) at 48 United States schools between 1994 and 1996

Type of symptom	Incidence	95% CI	Type of symptom	Incidence	95% CI	Type of symptom	Incidence	95% CI
Nasal drainage and congestion	19.8	±1.3	Discomfort complaints			When are symptoms the worst?		
Itchy or watering eyes	14.3	±1.1	Odorus	5.2	±0.4	High humidity	12.0	±0.9
Contact problems	5.6	±1.2	Temperature (hot/cold)	7.2	±0.1	Low humidity	0.0	±0.0
Headaches	12.5	±0.6	Noise	0.8	±0.3	Spring	3.9	±0.8
Sinus	10.3	±0.5	Ventilation	6.1	±0.3	Summer	0.0	±0.0
Severe	3.4	±0.4				Fall	2.7	±0.6
Increased airway infections	14.3	±1.0	Onset of symptoms			Winter	4.5	±0.5
Cough	6.5	±0.6	Entering the building	3.4	±0.9	Start of school	5.7	±2.0
Shortness of breath	5.9	±0.4	Working in the building	11.0	±1.7	Morning	3.4	±0.4
Sneezing	6.8	±1.0	Start of school	11.3	±1.9	Afternoon	1.1	±0.3
Dizziness	2.2	±0.5				Monday	0.8	±0.3
Fatigue	1.1	±0.3	When do symptoms go away?			Late in week	0.8	±0.3
Flu-like symptoms	1.8	±0.6	Never	3.5	±0 8	No pattern	1.1	±0.3
Nausea	1.8	±3.4	Leave work	2.1	±0.6	Always	2.3	±0.6
Allergies	17.0	±1.0	Weekends	4.3	±0.9	Before remedy		
Asthma	1.4	±0.3	Vacations	14.7	±2.5	IAQ complaints or symptoms	31.3	±6.8
Other health conditions	1.2	±0.5	Medications	4.4	±0.2	After remedy		
						IAQ complaints or symptoms	2.5	±1.1

air machines to prevent migration of particulates. The cleaning was conduned in accordance with the National Air Duct Cleaners Association Standard 1992-01.[24] All work was done after hours or at weekends. All personnel involved in the remedial work had the proper safety equipment and training and the Occupational Health and Safety Administration standards were observed. Air and swab samples were retaken within 60 days and after at least 6 months of completion of remedial work.

DATA ANALYSIS

Data were analysed by a computer program (Sigma Stat) with the Mann-Whitney rank sums test (*U* rut), the Kruskal-Wallis one way analysis of variance (ANOVA) (*H* test), Spearman's product moment correlation, and Dunn's multiple comparisons and partial correlations.[25]

Results

COMPLAINTS

Of the 48 schools surveyed, 40 were elementary schools (children aged 5–10). At most of these sites the school nurse distributed the IAQ company's questionnaire to only the staff. There were 622 occupants that reported IAQ symptoms or complaints, which represented 28% of the total staff (students were not included). With the exception of nausea, there were no significant differences between the reported complaints and symptoms at the different sires. All of the sires were combined and the average incidence per 100 employees along with the 95% confidence intervals (95% CIs) are displayed in the cable. Nasal drainage and congestion (IR 19.3, 95% CI ±1.3) and itchy and watering eyes (IR 14.0, 95% CI ±1.1) were always the most common complaints, although all of the symptoms, (with the exception of nausea) listed in the table were reported at each site. Most of the occupants registering complaints stated that their symptoms were a result of either entering or working in the building and most stated that the symptoms usually went away during weekends and holidays and returned on entering the building. More than half of the occupants that had IAQ complaints also complained of increased respiratory infections (such as tonsillitis, bronchitis, and some cases of pneumonia) (IR 14.3, 95% CI ±1.0). Over one third of the occupants that registered complaints claimed that an increase in the relative humidity resulted in an increase in the severity of their symptoms (IR 12.0, 95% CI ±0.9).

At moat schools, before the IAQ company's investigations, public health departments had conducted investigations and used questionnaires. These investigations consisted primarily of measuring the CO_2 in the buildings and increasing the ventilation rates, but with no success in reducing the IAQ complaints. The symptoms from the public health departments' questionnaires correlated with the symptoms obtained from the IAQ company's questionnaire. At all schools, many of the occupants that had IAQ complaints had submitted letters to the school officials, detailing their complaints and symptoms. The complaints and symptoms listed in these letters correlated with the complaints and symptom obtained from the IAQ company's questionnaire.

After remedial action to any building, questionnaires were made available to the staff. They were asked to say if there were any complaints or symptoms related to the IAQ of the building. There were none or only a few complaints concerning the IAQ hum the buildings that underwent remedial action. Complaints after the action were never registered by more than 3% of the staff at any school. The overall incidence was 2.5/100 employees (95% CI ±1.1). This represented a significant reduction ($p<0.001$) in the number of IAQ complaints.

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CARBON DIOXIDE CHEMICAL AND PARTICULATE MEASUREMENTS

Although CO_2 concentrations were higher indoors than outdoors, there were no significant correlations between the indoor concentrations and the complaints or symptoms (data nor shown). All measured constituents were well within the normal acceptable range of a school or office and there were no significant correlations between the indoor concentrations and complaints or symproms (data nor shown). No correlations were found between the outdoor particulate measurements, the indoor complaint areas, and the non-complaint areas.

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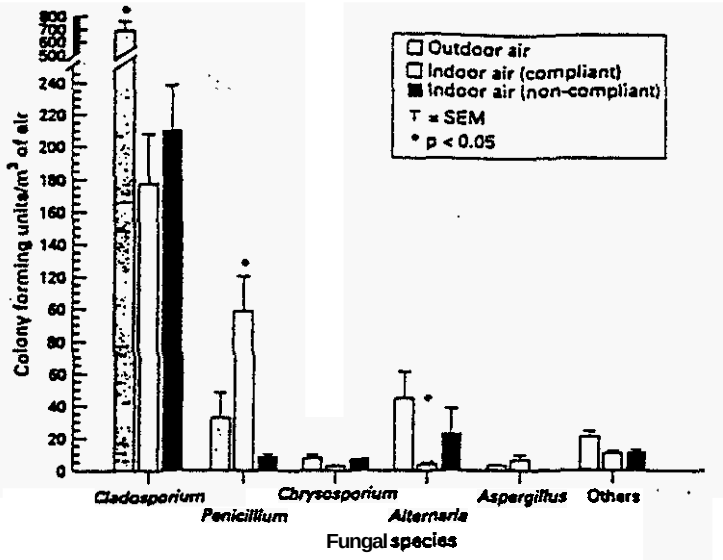

Figure 1 Bar graph of all air samples taken at the 48 schools.

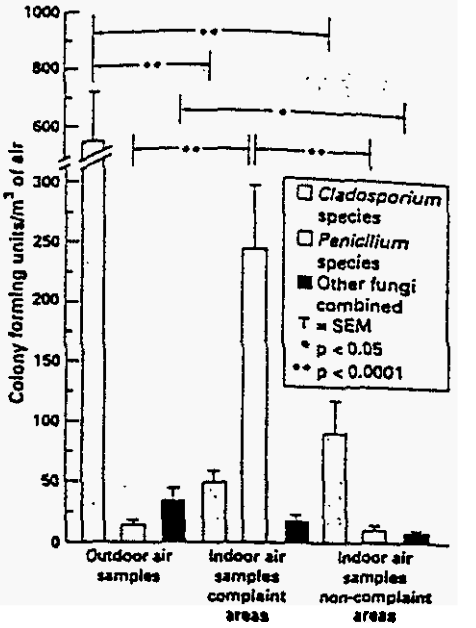

Figure 2 Bar graph of all air samples taken at the 20 schools where Penicillium species were the dominant fungi.

FUNGI IN OUTDOOR AIR

Five fungal genera were consistently found in the outdoor air and comprised over 95% of the outdoor fungi (fig 1). These genera were *Cladosporium* (81.5%), *Penicillium* (5.2%), *Chrysosporium* (4.9%). *Alternaria* (2.8%), and *Aspergillus* (1.1%). Other fungi (*Fusarium, Epicoccum, Botrytis, Bipolaris, Acremonium, Drechslera, Rhizopus, Mucor,* and *Rhodotorula*) were present in very low numbers and varied according to location and season. The predominant *Cladosporium* species isolated from the outdoor samples waa *Cladosporium cladosporioides*. Several other *Cladosporium* species (such as *Cladosporium herbarum* and *Cladosporium sphaerospermum*) were also isolated, but these isolates were usually found in association with *Cladosporium cladosporioides*. The predominant *Penicillium* species isolated was *Penicillium chrysogenum*. *Aspergillus niger* was the most commonly isolated *Aspergillus* species from outdoor air samples. With the exception of a few sites along the northern Atlantic coast, most of the buildings were in mild temperate zones, with little or no snowfall, and an average relative humidity range of 30%–60%. The rainfall in the survey areas was not abnormal, with the exception of states surrounding the southern region of the Gulf of Maico, which were experiencing a drought during the survey period. The outdoor temperature was seasonal, varying from a low near 5°C to a high of 38°C.

INITIAL INDOOR AIR SAMPLES

In most af the schools (fig 1), there were significantreductions in the CFU/m' of fungi in the indoor air samples from non-complaint areas compared with the outdoor air sample, but the fungal profiles were similar to outdoor air. In all of the buildings, the CFU/m' of *Cladosporium* species were. significantly ($p<0.05$) lower in the indoor air samples of non-complaint areas than in the oucdwr air sample. In the indoor air sample complaint areas, the CFU/m' of *Cladosporium* species were lower, but *not* always significantly lower, than the outdoor air sample. *Penicillium* species and *Aspergillus* species were the only fungi isolated from the indoor air sample complaint areas that had higher CFU/m' of air when compared with the outdoor air sample and indoor air sample non-complaint areas. All of the schools had similar interior temperatures (23°C). Most complaint sites showed very little HVAC maintenance as well as active water leaks.

At 20 schools (fig 2), there were significant increases ($p<0.0001$) in the CFU/m' of *Penicillium* species in the indoor air samples of complaint areas compared wirh the outdoor air sample and the indoor air samples of non-complaint arcas. The swab samples from these sites had very heavy growth of *Penicillium* species. The mean (SD, range) indoor relative humidicy in these complaint areas (IRH-C) was 50% (12%, 23%–67%), in non-complaint areas (IRH-NC) 40% (10%, 30%–48%). The mean (SD, range) outdoor relative humidity (ORH) was 46% (20%, 22%–81%). *Penicillium chrysogenum* was the dominant fungal isolate in 14 of these sites.

In the air samples from complaint areas at five schools (data not shown) there increases, although nor significant ($p=0.10$), in the number of CFU/m' of *Penicillium* species. In these the IRH-C had a mean (SD, range) of 64% (9%, 54%–70%), the IRH-NC 56% (3%, 54%–58%). The outdoor relative humidity had a mean (SD, range) of 69% (15%, 60%–86%). The swab samples from these sires showed very heavy growth of *Penicillium* species and heavy growth of *Cladosporium* species, which indicated partible fungal growth in the interior and a potential IAQ problem. *Penicillium chrysogenum* was the most common isolate.

At 11 schools (data not shown), *Stachybotrys atra*, which was not isolated from any air samples, was isolated from swabs of the visible growth taken from under wetted carpets, interior painted gypsum board walls, and especially behind vinyl coverings on gypsum board walls that had been wetted in indoor complaint areas. The indoor air samples from these complaint areas were not significandy different from the indoor air samples of the non-complaint areas and had profiles that were similar m the outdoor air samples. The mean (SD, range) of these IRH-C samples was 62% (5%, 58%–66%). The mean (SD, range) outdoor relative humidity was 83% (11%, 65%–90%).

Three *Aspergillus* species (*Aspergillus glaucus*, *Aspergillus versicolor*, and *Aspergillus flavus*) were isolated from interior air samples and swab samples in association with the *Penicillium* species, but *Aspergillus* species (*Aspergillus flavus*) were dominant in complainr *MY* at only one school. The swab samples from the interior showed heavy to very heavy growth of *Aspergillus* species, along with medium to heavy growth of *Cladosporium* species and *Penicillium* species. The indoor relative humidity at this site was 65% and the outdoor relative humidicy was 75%.

In the remaining 11 schools, the fungal ratios and CFU/m³ of air (outdoor and indoor air samples) were not significandy different. The swab samples from the interior of these sites showed heavy to very heavy growth of *Cladosporium* species or *Penicillium* species, which indicated possible fungal growth in the interior. The mean (SD, range) indoor relative humidiry was 60% (3%, 56%–64%) and outdoors it was 60% (2%, 57%–62%).

INDOOR AIR SAMPLES AFTER REMEDIAL ACTION

Indoor air samples and swab samples were retaken within 60 days of completion of the remedial action to a building and again at least 6 months after the action. At all sites the fungal ratios (outdoor air samples compared with indoor air samples) were wry similar but the fungal CFU/m³ from the indoor air samples were 50%–90% less ($p<0.05$) dun the outdoor air samples (data not shown). The indoor relative humidity had a mean (SD) of 44% (5%), with no site exceeding 57%. The outdoor relative humidiry ranged from 37%–87%. All swab samples showed very light to light growth of *Cladosporium* species and a mixture of other species.

Discussion

All of the IAQ complaints investigated were generated by the occupants. As the validity of results from questionnaire studies may be altered by biases introduced by the observer or by the respondents,[27] the results must be carefully weighed. Even when observer bias is reduced, the bias introduced by the respondent remains a potential source of systematic error.[28] A major problem, however, is that it is often difficult to control independent variables because of the diversity of the study population, its motility, or a lack of personal exposure. Most of the occupants (90%) in the buildings that we investigated were teachers. Although potential psychological disorders—such as depression or anxiety—were not directly considered, we did, at most schools, find a high job satisfaction and a genuine concern for the welfare of the students. Also, it is difficult to segregate the individual pollutant from copollutants and other confounders. However, tobacco smoke can be eliminated as a potential confounder as all of the schools prohibited tobacco use on the campuses.

A causal relation is rarely discernible even with strong statistical significance. Thus, at best, associations can be drawn only between the exposure and the effect.

Although fungal contamination in indoor environments has been shown to produce allergies in occupants of these buildings,[16 18 27 28] the role of fungi in IAQ problem has become increasingly controversial. Our studies show that *Penicillium* species and *Stachybotrys* species are strongly associated with SBS. These data show that the *Penicillium* species, especially *Penicillium chrysogenum*, can adapt to an environment in which humans are most comfortable. Our studies also support earlier findings that *Penicillium* species has become an important indoor contaminant.[29] This ubiquitous organism's optimal growth occurs between 10° and 25°C. It can grow o w a wide range of water availability and has low water activity, although sporulation requires a higher water activity.[30] Whereas it is widely stated that relative humidity >70% is needed for active fungal growth, the water activity of the substrate is actually the critical variable.[31] In the complaint areas where *Penicillium* species were dominant, we found (with the exception of the HVAC system at the fan during cooling) that the range of the indoor relative humidity was from 23%–67%. *Penicillium chrysogenum* is apparently capable of successfully competing with most conidial fungi over almost the entire range of water availability. Its spores are small (1–5 μm) and are capable of entering the lower respiratory tract. It has been shown that bronchial challenges with *Penicillium* species spores induced immediate and delayed type asthma in sensitised subjects.[32]

Stachybotrys species, some of which are capable of producing potent mycotoxins,[33] require abnormally high relative humidity or wetted surfaces to grow. This fungus has been associated with illness related to buildings and SBS."[34] It is difficult to isolate *Stachybotrys* species from the air and the presence of *Stachybotrys* may have been overlooked due to this phenomenon. Our findings suggest that when the fungal ecology of complaint air samples are similar to the outdoor air and non-complaint air samples, coupled with abnormally high interior relative humidity and symptoms of SBS, the possibility exists that a mycotoxin producing fungus such as *Stachybotrys*, may be hidden and growing in the interior of the building.

Spores of *Cladosporium* species probably occur more abundandy world wide than any

other spore type and are the dominant airborne spores in many areas, especially in temperate climates.[15 16] Similar to other studies, we found *Cladosporium cladosporioides* growing inside buildings on various building materials.[17 18] Although our findings showed that *Cladosporium cladosporioides* was not associated with the indoor air sample complaint areas, its presence indicated that the conditions favoured fungal growth that could potentially allow fungal genera such as *Penicillium* or *Aspergillus* to become the dominant organism.

The underlying factor for SBS is the modern, waled building with its environment controlled by an HVAC system. These systems probably contribute to the onset of SBS by allowing build up of pollutants when the capacity of the HVAC system is inadequate or has been compromised. Our finding suggests that rhe initial stage of interior microbial growth began with water leaks that wetted various building materials. If these wetted materials are not properly mitigated, fungal growth may occur. Eventually, the HVAC system becomes contaminated. Although an understanding of the pollutants or conditions directly responsible for SBS is essential to developing strategies for prevention, a thorough analysis of the HVAC system, along with removing or properly repairing wetted areas, were often the key to mitigating the problem in a particular building.

After remedial action, the average air change per hour was 0.5. The air samples taken at 60 days and again at 6 months after remedial action had at least a 50% reduction in the number of CFU/m³ from the indoor air sample than the outdoor air sample. For particles with an aerodynamic sue 32.5 µm, the current evidence suggests that 1.0 air change per hour results in indoor concentrations of about 30%–80% of that outdoors.[19]

These findings show that remedial action to the buildings removed interior fungal growth. With a significant reduction m complaints about IAQ from occupants, our data suggest that *Penicillium* (*Penicillium chrysogenum*) species and *Stachybotrys* species may be strongly associated with SBS.

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Endotoxin Exposure as a Major Determinant of Lung Function Decline in Pig Farmers

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Exposure-response relationshipr tor endotoxin ge measured in dust and longitudinal decline in lung function were studied. A cohort of 171 pig farmers was followed over a 3-yr period. Long-term average exposure to dust and endotoxin was determined by personal monitoring in summer and winter, using data on farm characteristics and activities. Mean decline in FEV_1 was 73 ml/yr and in FVC 55 ml/yr. Long-term average exposure to dust was 2.63 mg/m³ (geometric SD [GSD] 1.30), and to endotoxin, 105 ng/m³ (GSD 1.5). Annual decline in FEV_1 was significantly associated with endotoxin exposure. An increase in exposure with a factor 2 was associated with an extra decline of FEV_1 of 19 ml/yr. Vogelrang PFJ, van der Gulden JWJ, Folgering H, Kolk JJ, Heederik D, Preller L, Tielen MJM, van Schayck CP. Endotoxin exposure as a major determinant of lung function decline in pig farmers.

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Although the increase in diagnosed asthma in recent decades is real enough (1), most of this increase remains unexplained. Rcccntly, it was stated that there is an urgent need to understand environmental influences bearing on the development of asthma (2). The author stressed that occupation is not only important as a direct cause of asthma but also as a valuable model for investigating potential interactions in its causation. When studying chronic disease in the population at large, knowledge of the relations between exposure and disease in specific occupational groups is important. This is especially the case for types of exposure that can be found in many situations. In the case of chronic respiratory disease, attention is focusing, more and more on the role of bacterial endotoxin, both in large number of work situations (3–7) and in the home environment (8). A number of cross-sectional studies reported associations between exposure to endotoxin as measured in dust and baseline lung function (3–7, 9–11) and/or changes in lung function across a work-shift (12–17). In several experimental studies short-term effects of controlled exposure to endotoxin in purified form (LPS) were shown, demonstrating that it has a strong potency to induce inflammation in the airways (18–22). An association between endotoxin exposure and lung function decline was reported by Schwartz and colleagues (23) in a 2-yr follow-up study in farmers. It remains unclear from this study at which levels of exposure effects were observed, nor was an exposure-response curve published. Bias from various sources makes it difficult to interpret cross-sectional studies reporting associations between endotoxin exposure and lung function. For a correct assessment of the effects of such exposure, it is imperative that data from longitudinal studies become available. The present study evaluates the association between endotoxin exposure, as measured in dust, and longitudinal decline of lung function in a 3-yr follow-up in a cohort of 171 pig farmers.

METHODS

Study Population

In 1990, a questionnaire survey was held among 1,504 pig farmers (24). To be able to study exposure-response relations, a study population was formed with sufficient contrast in respiratory morbidity, and therefore presumably also in exposure. In 1991, 200 randomly selected subjects from among the respondents with one or more chronic respiratory symptoms (chronic cough, chronic phlegm, shortness of breath, ever wheezing, frequent wheezing, chest tightness/asthma), and 199 randomly selected subjects without such symptoms were invited for a medical examination that included an extensive interview. Of the 200 farmers who reported one or more chronic symptoms in the postal questionnaire, 115 qualified for the category of chronic obstructive pulmonary disease (COPD) and or asthma, on the basis of further information obtained in the interview at the medical examination. They were considered to be consistently symptomatic. Of the 199 farmers without chronic symptoms in the postal questionnaire, 145 revealed no chronic symptoms either in the more extensive interview. They were considered to be consistently asymptomatic. Ninety-eight of the consistently symptomatic and 100 of the consistently asymptomatic were randomly selected for a follow-up program which included measurements of environmental and personal exposure in their own confinement units. All worked at least 5 h d in pig farming. This cohort was invited in June 1992 (25) and June 1995 for medical examinations which included a measurement of lung function. In 1995, participants again completed a questionnaire on symptoms. The number of farmers that participated on both occasions was 171 (82 of the consis-

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tently symptomatic and 89 of the consistently asymptomatic). Reasons for nonparticipation in 1995 were mostly "no time/too busy" or "no interest any more." One farmer had died from a nonpulmonary disease.

Lung Function

Lung function was measured in 1992 with a Vicatest dry rolling-seal spirometer and in 1995 with a Vicatest (n = 94) or a Sensormedics Pulmonet3 water-sealed spirometer (n = 77). All measurements were taken according to European Respiratory Society (ERS) guidelines (26), and corrected to body temperature and pressure, saturated with water vapor (BTPS). The study was approved by the Committee for Ethical Research of the University of Nijmegen.

Exposure

Measurements of exposure were carried out on the farms of all participants during full work-shifts of on average 8.3 h on 2 d in summer 1991 and winter 1992. Personal exposure to inhalable dust was determined using a dust sampler with a 6-mm diameter inlet opening and an airflow of 2 L/min. Endotoxin in the inhalable dust samples was analyzed with a modified kinetic Limulus Amoebocyte Lysate test (27). Methods for measurement of dust and endotoxin are described in detail elsewhere (28, 29). Because the day-to-day variations of exposure to dust between days in individual participants were considerable compared with the variations of exposure in the entire group, the long-term average exposure was predicted by a mathematical modeling technique: long-term average exposure to dust and endotoxin of each individual farmer was estimated using data on farm characteristics and time spent on activities in pig farming of all cohort members combined. This is described in detail elsewhere (30). From the total number of 171 cohort members, complete data on exposure to dust and endotoxin became available for 146 participants.

Analysis

As exposure was lognormally distributed, logtransformed values with base 2 were used in the analyses of associations with health effects. Declines in lung function were normally distributed. Associations between exposure and lung function decline were tested with linear regression analysis, adjusting for age, baseline FEV_1 (baseline FVC for analyses with FVC), and smoking behavior, defined as pack years of cigarettes. For current and past cigarette smokers the numbers of pack years were calculated by the number of cigarettes smoked per day multiplied by the number of years smoked, divided by 25.

One participant contracted severe pulmonary disease in the follow-up period, resulting in a 2.5 liter loss of lung function. To avoid distortion of the results, he was excluded from the analyses of associations with exposure.

Computations were completed with Statistix for personal computers.

RESULTS

Personal Characteristics

Mean age of the 171 farmers was 39.6 yr at the start of the observation period (Table 1). Mean number of years worked in pig farming was 16.7. Five participants were no longer active as pig farmers in 1995, three of them stopped partly because of respiratory problems. Percentage of cigarette smokers was 25.

TABLE 1

PERSONAL CHARACTERISTICS, LUNG FUNCTION, AND ESTIMATED LONG-TERM AVERAGE EXPOSURE OF 171 PIG FARMERS DURING THE 3-yr FOLLOW-UP BETWEEN 1992 AND 1995

Mean age, yr (standard deviation)	39.6	(9.6)
Number of years working as pig farmer, yr (SD)	16.7	(8.2)
Number that stopped as pig farmer during follow-up	5	(3%)
Number of smokers in 1992	52	(25%)
Lung function in 1992		
FEV_1, L (SD)	3.97	(0.90)
FVC, L (SD)	5.06	(0.98)
Mean annual decline		
FEV_1, ml (SD)	73	(97)
FVC, ml (SD)	55	(112)
Exposure (geometric mean, geometric standard deviation)		
Inhalable dust, mg/m^3	2.63	(1.30)
Endotoxin, ng/m^3	105	(1.5)

Lung Function

Mean FEV_1 and FVC of the 171 participants was 3.97 L and 5.06 L, respectively, in 1992 (Table 1). Farmers with chronic symptoms in 1991 had a significantly lower lung function than those without. Mean decline in lung function was 73 ml/yr for FEV_1 and 55 ml/yr for FVC. This was not different for farmers consistently symptomatic or asymptomatic in 1991.

Decline in FEV_1 was smaller for those tested with the water-sealed spirometer in 1995 than for those tested with the dry rolling-seal spirometer on both occasions. After leaving out one participant who had a difference of 2.5 L in his lung function, the mean decline was 57 ml/yr (SD 68) versus 79 ml/yr (SD 82), $p = 0.06$.

Exposure and Associations with Respiratory Effects

Estimated long-term average exposure to inhalable dust was 2.63 mg/m^3 (geometric mean) and to endotoxin this was 105 ng/m^3 (Table 1).

After adjusting for age, baseline FEV_1 or FVC and smoking, decline in FEV_1 during the 3-yr period between 1992 and 1995 was significantly associated with endotoxin exposure alone, whereas decline in FVC was associated with both endotoxin exposure and inhalable dust (Table 2). The association between endotoxin exposure and annual decline in FEV_1, based on these data, is represented in Figure 1. Over the whole range of exposure to endotoxin in this group, annual decline in FEV_1 varied from 40 ml for the lowest exposed farmers to 100 ml for the highest exposed ones.

The associations between exposure and lung function decline were stronger for the group tested with the dry rolling-seal spirometer on both occasions than for the group tested with the water-resled spirometer in 1995 (data not shown).

DISCUSSION

In this 3-yr follow-up of 171 pig farmers, we showed a large decline in FEV_1 and FVC. Long-term average exposure to endotoxin was found to be associated with decline of FEV_1 and FVC, whereas exposure to dust was associated with decline of FVC alone.

The mean annual decline of FEV_1 of 73 ml is large compared to the expected age-related decline of 29 ml/yr (26), and equal to that of 73 ml in pig farmers reported by Iversen and

TABLE 2

ASSOCIATIONS BETWEEN ^{2}LOGTRANSFORMED LONG-TERM AVERAGE EXPOSURE TO ENDOTOXIN AND INHALABLE DUST AND DECLINE IN LUNG FUNCTION OF 171 PIG FARMERS DURING THE 3-yr FOLLOW-UP BETWEEN 1992 AND 1995*

		Regression Coefficient	Standard Error	p Value
FEV_1 decline, ml/yr	Endotoxin	19.4	10.9	0.04
	Dust	7.4	16.0	0.32
FVC decline, ml/yr	Endotoxin	40.7	14.2	0.002
	Dust	41.2	21.0	0.03

Adjusted for age, baseline FEV_1 or FVC and smoking (pack years of cigarettes). Number of pig farmers in the final regression model = 142.
* Tested one sided.

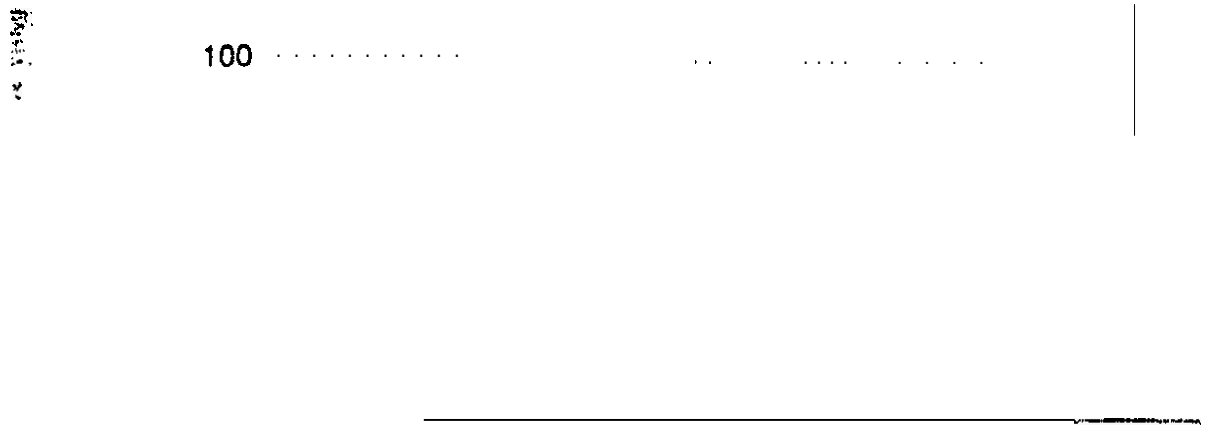

Figure 1. Predicted association between log*transformed* endotoxin exposure **and** annual **rate** of decline **of** FEV_1 (with standard **error**), corrected **for** age, baseline FEV_1, and pack-*years* **of** smok ng. Based **on** 171 **pig farmers.**

colleagues **(31).** The selection procedure did not affect this figure, as decline was similar fur farmers with or without symptoms. This it probably due to the fact that **at** the start of *follow-up symptomatic* farmers had **a** mean **lung** function **only** slightly different **from** normal. The group tested with a different spirometer at the **end** of the follow-up. showed **a** smaller (though still large) mean decline than the group tested with the same spirometer on both occasions. **As** these groups were not different in any relevant feature **at** the start of the **study. we assume that** the **true** decline in FEV_1 is higher **than** the **73** ml/yr on **average** found.

Long-term average exposure to endotoxin was **an** important factor associated with this decline. Logtransformed **exposure with** hare 2 was used in analysis. This **means** that **an** increase in exposure with **a** factor 2 **was** associated with an **extra** decline *of* FEV_1 **of** 19 ml/yr. In multivariate **regression**, pig farmers with the **lowest** levels **of** exposure showed **an** average predicted decline similar **to** the expected age-related decline. whereas average predicted decline **of** the highest exposed pig farmers was **3s** much as 100 ml per year. Part of the group was tcsrcd *with a different spirometer* in 1995. *This* probably *introduced* some underestimation **of** the exposure-response relationship: associations were stronger **in** the subgroup tested with the same spirometer both in 1992 and 1995. Modeled long-term average exposure was used rather than the mean of the two measurement days per farmer. Exposure assessment taking repeated samples at each farm would have required at least 30 measurements per farm to obtain a reliable estimate of the long-term average exposure. This is because of the large variation in dust and endotoxin exposure from day to day, when compared with the differences in exposure between farms. *We applied a recently described method* (30) that predicted exposure, using information on farm characteristics (e.g., type of floor and feeding method) and tasks (e.g., controlling, cleaning, teeth cutting). A small validation study was performed among six farmers for which six to nine valid monthly measurements were available. This showed that the estimated long-term average exposure *gave a better reflection of the long-term* **exposure** (mean of monthly measurements) than did the average of the **two** measurement days available for the entire group (30).

In *cross-sectional* studies. *adverse effects of endotoxin* exposure **on** baseline lung function have been shown before in **pig** farmers (3, 5, 11)and other occupational groups (4, 6, 7.9. 10, 13). One **study** group reported an association between endutoxin exposure and increased loss of lung function **in** a longitudinal observation **of** 2 yr on average (23). However, in that study **no** clear associations between levels of exposure snd rates of decline in lung function were shown. This is **the** first study that shows a clear exposure-response relation of endotoxin with longitudinal decline of lung function. Occupational threshold exposure levels of 100 and **30** ng/m^3 **(32,** 33) have been proposed, to prevent respiratory disease. **The** health effecuseen in our study support the lower **of** there proposals.

The balance **of** studies on pig farmers suggests **development** of obstructive rather than restrictive pulmonary disease (31, 34). In studies in various occupational circumstances **en.** dotoxin exposure correlated better with (changes **in**) lung function wkcn *compared with dust exposure* (3, 5, **7. 10.** I). In our study exposure to inhalable dust correlated with lung function decline measured as FVC, but not with FEV_1, whereas endotoxin correlated with both. **Although** inhalable dust is a more **generally** available and cheaper marker fur exposure, we conclude **that** dust **exposure** alone is insufficient as predictor lor **lung** function decline in pig farmers. We established **a** clear link to exist in a longitudinal study between lung function and occupational endotoxin **exposure** in **a** basically healthy group of workers. As strong effects were demonstrated in healthy workers **at** levels previously not proved harmful, it becomes **increasingly** important also to know the role played by endotoxin axpusure in the development of respiratory disease in a domestic environment. There is evidence that susceptible pupa. especially asthmatics, can be affected **by** chronic exposure at lower levels. Michel **and** colleagues confirmed that natural exposure to endotoxin in house dust **in** related to the clinical status *of asthmatics* (8). Sparse information available on endo-

toxin exposure in domestic environments indicated airborne levels of up to 1.2 ng/m^3 (35), much lower than in the working situation. Research is needed on relevant levels of exposure to endotoxin (airborne or in dust from beds or floors?) and the mechanisms by which exposure leads to respiratory disease.

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Highly Sensitive Protein Translation Assay for Trichothecene Toxicity in Airborne Particulates: Comparison with Cytotoxicity Assays

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Screening assays for environmental mycotoxins in bulk samples currently use cytotoxicity in cell cultures, but their application to air particulate samples often lacks sensitivity and specificity for fungal spores. An assay based on inhibition of protein synthesis using translation of firefly luciferase in a rabbit reticulocyte system has been developed lor the detection of trichothecene mycotorins found in the spores of toxigenic fungi. Ethanol extracts of air particulates trapped on polycarbonate filters are ultrafiltered and applied at several dilutions to a translation reaction mixture. The activity of translated luciferase is measured directly in a luminometer, eliminating the need for radioisotopes and time-consuming sample processing. Parallel standard curves using a commercially available trichothecene provide for expression of the results in T-2 toxin equivalents per cubic meter of air. The assay can be completed in 2 band is readily applicable to multiple samples. Comparison to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cytotoxicity assay indicates a 400-fold increase in sensitivity of trichothecene detection in addition to a much higher specificity for these toxins. Initial field testing indicates a strong correlation between the measured level of toxicity and the presence of toxigenic fungi detected with microbiological methods In conclusion, this luciferase translation assay offers a rapid and highly sensitive and specific method for quantitative detection of trichothecene mycotoxin activity in air particulate samples.

Because fungal viability may be short-lived compared to toxin stability, methods of detecting toxins or toxicity are much preferred over those requiring fungal culturing. Quantitative tests for airborne environmental fungi which are most widely used arc based on culturing of air particulates collected on filters and determination of the number of viable spores. The making of public health decisions would be greatly facilitated by the development of rapid and affordable strategies which provide accurate quantitative assessment of possible environmental exposure to fungal toxins.

Existing methods of trichothecene toxin detection include costly, highly technical approaches, such as gas chromatography or mass spectroscopy (23). Immunodetection requires specific antibodies which arc not readily available at the present time (8). Thin-layer chromatography has been used to detect mycotoxins, but its sensitivity is significantly lower than that of cytotoxicity measurements (25). Cell culture-bawd cytotoxicity assays (12, 14, 19) appear to work well with samples generated under controlled conditions, such as growing fungi on sterile substrates, but interpretation of the results becomes problematic when environmental bulk samples are studied. In addition to fungi, those samples arc mmmonly heavily contaminated by bacteria, which raises the possibility of various synergistic effects of mycotoxins and other substances, such as endotoxin. Specificity may be less of a problem in the application of cytotoxicity assays to airborne particulates, hut the sensitivity of these tests would preclude quantitative evaluation (22). In an attempt to use this approach with airborne particulates, wc have tested swine kidney cells by using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (14). We have found that cytotoxicity assays, while yielding reproducible results. lack both the specificity and sensitivity needed to detect *mycotoxins* in fungal spores collected on filters in indoor air sampling using low (3 liters/min)-to-medium (22 liters/min)-flow pumps. For cytotoxicity assays, either high-Paw pumps or prolonged collection periods are neededto sample the large quantities of air required.

Mycotoxin detection based on the inhibition of protein synthesis has been described by others (28). Toxin detection and methods used in studying the mechanism of action at the protein translation level (27) have relied on the u s of radioisotopes and vary in sensitivity to 12,13-epoxytrichothecenes. Although they can have serious limitations, bioassays based on inhibition of protein synthesis demonstrate high specificity and sensitivity toward trichothecene mycotoxins. Wc have developed a nonradioactive assay based on translation .of firefly luciferase in a rabbit reticulocyte system and have compared its sensitivity and specificity with those of the MTT assay by using both pure mycotoxins and air particulate samples collected from fungus-contaminated houses

MATERIALS AND METHODS

Materials and reagents. PK15 cells were obtained from the American Type Culture Collection, Manassas, Va. DON (deoxynivalenol) and T-2 toxin were purchased from Sigma, St. Louis, Mo. Satratoxin G was a generous pill from Bruce Jarvis, University of Maryland. The rabbit reticulocyte lysate, luciferase mRNA, amino acids, magnesium acetate, potassium chloride, recombinant RNase inhibitor, and luciferase assay reagent used were from Promega, Madison, Wis. RNase T-1 from *Aspergillus oryzae* was from Gibco BRL, Gaithersburg, Md.

Collection and processing of air samples. Air samples from fungus-contaminated houses, as well as clean control rooms, were collected on polycarbonate filters (pore size, 0.8 μm; Poretics Corp., Livermore, Calif.) using low-flow (3 liters/min for 24 h) and medium-flow (18 and 22 liters/min for 8 h) pumps. Filters

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were extracted overnight in 10 ml of 95% ethanol and sonicated for 30 min. Another 5 ml of ethanol was added to the filter, and sonication was repeated for 30 min. Extracts were passed through 0.22-μm-pore-size GV Millex (Millipore Corp., Bedford, Mass.) filters to remove particulates and evaporated. Ethanol was the solvent of choice because of its compatibility with the filters used to remove endotoxin and RNase. Dried samples were reconstituted in small volumes of ethanol and appropriately diluted with buffer or culture medium for testing. For cytotoxicity studies, samples were filtered through Ultrasart D20 (Sartorius, Goettingen, Germany) to remove endotoxin. Extracts used in translation inhibition assays were passed through Millipore Ultrafree-MC 5000 NMWL filter units to remove proteins.

Bulk samples and isolated fungal spores. *Stachybotrys chartarum*, originally isolated from a home in Cleveland, Ohio (JS5817; American Type Culture Collection catalog no. 201211), was grown in culture on rice. Rice (100 g) was suspended in 60 ml of distilled water and allowed to stand for 1 to 2 h before autoclaving. The rice was sterilized by autoclaving, inoculated with suspensions of 7-day-old conidia, and incubated at 28°C for 4 weeks. Additional water (5 ml) was added axenically after 48 h of incubation. Cultures were stored at 4°C until needed. Small portions of rice culture (volume not important) were shaken into a small plexiglass chamber (8 by 8 cm [internal dimensions]) provided with two openings. The chamber had previously been disinfected with 70% isopropanol. Incoming air was filtered through sterile glass wool in a 37-mm filter cassette, and the air-entrained spores were collected on an open-faced 37-mm cassette connected to an external vacuum source. The chamber was hand shaken to aerosolize the spores within the chamber, and the entire operation was performed in a chemical fume hood. When the collection filters were completely black, the vacuum was stopped, the chamber was disassembled, and the filters were transferred to sterile 50-ml centrifuge tubes for transport. The operation was repeated until it was no longer possible to collect spores from the rice. Samples from the filters were examined microscopically for the presence of hyphae and/or conidiophores and tested for fungal and bacterial contamination by streaking on malt extract agar and incubation in trypticase soy broth, respectively. Filters containing spores were rinsed with 1 to 2 ml of phosphate-buffered saline. Spores released from the filter were enumerated microscopically with a hemocytometer. Known numbers of spores were then pelleted by centrifugation and extracted with ethanol as described above. Bulk samples (fragments of wallpaper and drywall and samples of dust, carpet fibers, etc.) were weighed prior to the extraction procedure. Extraction was performed as described for filters.

Identification of fungal species in residences with water problems. Bulk samples collected in residences (surface samples such as dust, wallpaper, etc.) were cultured under standard conditions on potato dextrose agar and Rose Bengal plates for 1 to 3 weeks at 30°C. Fungi were identified based on their morphology.

Cell culture. PK15 porcine kidney cells were cultured at 37°C in Eagle's minimum essential medium with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, Earle's balanced salt solution, and 5% newborn calf serum antibiotic free in an atmosphere of 5% CO_2. For cytotoxicity measurements, cells were trypsinized (0.25% trypsin, 0.03% EDTA) and passaged onto 96-well plates at a density of 5×10^5/ml and a volume of 150 μl/well. After 24 h, confluent cells were exposed to toxins and extracts for 72 h prior to MTT assay.

Preparation and storage of trichothecene mycotoxins and air particulate extracts. Concentrated stocks of T-2 toxin, satratoxin G, and DON (1 mg/ml) were prepared in ethanol to ensure their complete solubility and subsequently diluted in the culture medium used to grow porcine kidney cells as described above just prior to their addition to the cultures. The final concentration of ethanol did not exceed 1% in the culture medium and was kept under 0.05% in the translation reaction mixture. Storage of trichothecenes and filter extracts in aqueous solutions was avoided at all times to prevent the loss of toxin activity observed by others (28).

MTT assay. MTT assays (20) were performed as described by Hanelt et al. (14) by using a Bio-Rad 3550 plate reader.

Translation of firefly luciferase mRNA. The translation reaction was carried out with 33% rabbit reticulocyte lysate, 0.25 mM magnesium acetate, 110 mM potassium chloride, 8.3 ng of luciferase mRNA per μl, 0.33 U of rRNasin RNase inhibitor per μl, an 8.3 μM amino acid mixture, 4 mM dithiothreitol, and diluted toxins or extracts in a final volume of 1 to 20 μl. Using smaller volumes allows one to save reagents but is technically difficult when using manual pipetting. Following incubation at 30°C for 90 min, samples were rapidly frozen on dry ice. The luciferase translation assay has to be performed with great caution to avoid the introduction of RNase from the laboratory environment. We routinely use sterile techniques, sterile glassware and plasticware, and RNase-free water and reagents.

Luciferase activity assay. The luciferase assay was performed following sample thawing and 20-fold dilution with 20 mM Tris/HCl (pH 7.8). Luciferase assay reagent (50 μl) containing 20 mM tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 μM coenzyme A, 470 μM luciferin, and 530 μM ATP (pH 7.8) was mixed quickly with 5 μl of the diluted translation mixture and read in an Optocompt I photon-counting luminometer (MGM Instruments Inc.). The activity of all samples was expressed as percent control (water added in place of toxin or extract). A purified luciferase preparation was used to choose the range of light intensity (relative light units [RLU]) proportional to the amount of luciferase present in the sample. Control samples (no toxin added) consistently yielded about 60,000 RLU, corresponding to 1 μg of luciferase per liter.

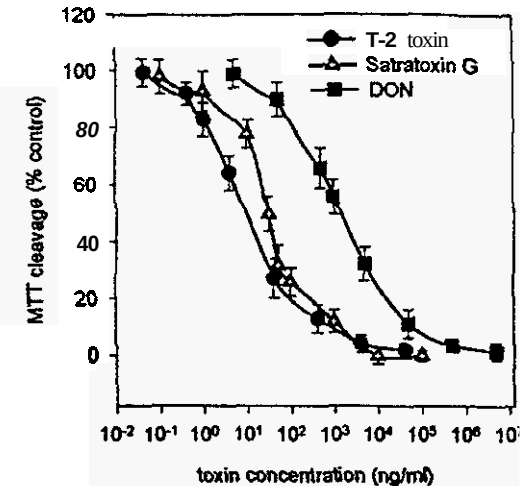

FIG. 1. Effects of T-2 toxin, satratoxin G, and DON on the MTT cleavage activity of PK15 cells. The values are means ± SEM ($n = 8$). T-2 toxin yielded 50% inhibition at 9.14 ng/ml ($r^2 = 0.98$), satratoxin G did so at 29.9 ng/ml ($r^2 = 0.98$), and DON did so at 1,470 ng/ml ($r^2 = 0.98$).

Data analysis. Dose-response curves were plotted and analyzed by using SigmaPlot and TableCurve programs (Jandel Scientific). Data from dose-response experiments were fitted into logistic dose-response equations, and 50% effective concentrations were calculated. Correlation coefficient (r^2) values were used to assess the goodness of fit and ranged from 0.950 to 0.999.

RESULTS

Cytotoxicity assays. Porcine kidney cells are highly susceptible to trichothecene mycotoxins, as demonstrated by Hanelt et al. (14) in a study comparing three different cell lines. We have found that swine kidney PK15 cells were more sensitive to trichothecenes and more resistant to solvents such as methanol and ethanol (no significant changes in MTT cleavage activity were detected at up to 5% ethanol) than the MRC-5 human lung fibroblast cells used by others for cytotoxicity measurements (19). Based on these findings, PK15 cells were chosen for further studies.

Three mycotoxins were tested for their cytotoxic effects on PK15 cells: two simple trichothecenes, T-2 toxin and DON, produced by *Fusarium* sp., which are commercially available, and a macrocyclic trichothecene, satratoxin G, isolated from *S. chartarum* (18). As shown in Fig. 1, T-2 toxin was found to inhibit MTT cleavage by 50% at a concentration of 9.14 ± 0.95 ng/ml. Satratoxin G produced the same effect at about three times the concentration (29.9 ± 2.6 ng/ml). DON was much less potent against PK15 cells, with 50% inhibition at 1.47 ± 0.12 μg/ml.

The cytotoxicity of air particulates collected in several homes with visible mold growth was evaluated by using material equivalent to 0.1 to 4.0 m^3 of air. Under these conditions, the MTT test did not reveal any cytotoxic effects of air particulate extracts (data not shown). Based on the sensitivity of the MTT test (see Table 2), the cytotoxicity of those samples was lower than that of 1 ng of T-2 toxin/m^3.

Bulk samples such as dust, fragments of carpeting, plaster, or wallpaper collected in houses with moisture and fungus problems were demonstrated to contain very high levels of cytotoxicity. While the level of cytotoxicity was significantly

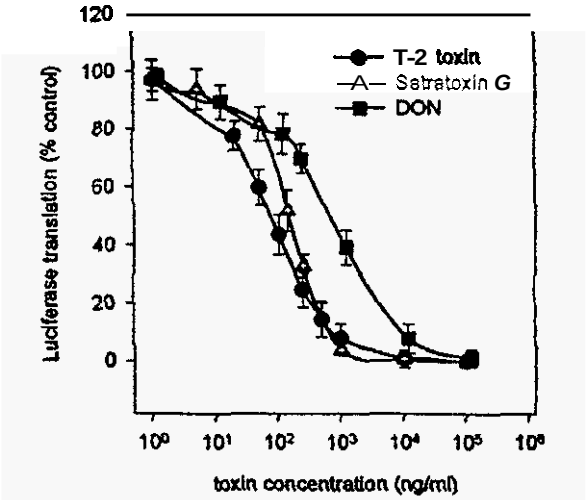

FIG. 2. Effects of T-2 toxin, satratoxin G, and DON on luciferase translation in rabbit reticulocyte lysate. The values shown are means ± SEM (n = 8). T-2 toxin yielded 50% inhibition at 78.5 ng/ml (r^2 = 0.99), satratoxin G did so at 148.5 ng/ml (r^2 = 0.99), and DON did so at 757 ng/ml (r^2 = 0.99).

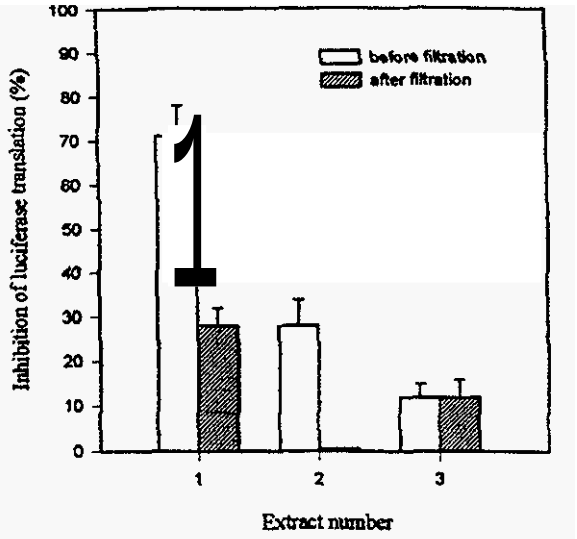

FIG. 3. Effect of filtration through Millipore Ultrafree-MC 5000 NMWL centrifuge filters on the protein translation inhibition activity of three different air particulate sample extracts. The values are means ± SEM (n = 3).

decreased by using filters which exclude bacterial endotoxin, e.g., Ultrasart D20 (data not shown), other toxic agents from paint, glue, or dyes that are highly soluble in ethanol are likely to be present.

Translation inhibition assay. The in vitro luciferase translation system was used to study the effect of trichothecenes on protein translation in a cell-free rabbit reticulocyte system. The standard reaction conditions for rabbit reticulocyte lysate (24) have been modified as described in Materials and Methods to reach high translation reaction efficiency and, at the same time, limit the use of reagents. Figure 2 shows the concentration-dependent inhibition of translation of firefly luciferase mRNA by T-2 toxin, satratoxin G, and DON. Similar to the cell culture-based system, there is a significant difference in the effect of DON (50% inhibition at 757 ± 43 ng/ml), satratoxin G (50% inhibition at 148.5 ± 7.7 ng/ml), and T-2 toxin (50% inhibition at about 7853 ± 13 ng/ml). Interestingly, T-2 toxin and satratoxin G are much less effective in this system than in PK15 cells.

The air particulate extracts from fungus-contaminated houses were strongly inhibitory in the rabbit reticulocyte system (Table 1). Control sterile filters showed no inhibition of luciferase translation. . . .

The content of extracts remained the main concern with this highly sensitive assay, especially with respect to RNase, which can be a serious problem in translation-based applications. The recombinant inhibitor that inhibits the RNases A, B, and C included in the reaction mixture is not effective against many bacterial and fungal RNases (3).

To determine whether the observed inhibition can be attributed to RNases or trichothecenes, the extracts were filtered through Millipore Ultrafree-MC 5000 NMWL centrifuge filter units with a molecular weight exclusion limit of 5,000. This procedure should remove proteins, most importantly, RNases and proteases. As shown in Fig. 3, such filtration of extracts of environmental air particulates leads to reduction of inhibitory activity. This suggests that these extracts contain significant levels of RNases or other high-molecular-weight compounds that interfere with the translation process or destroy the translation product (proteases). The concern about RNase interference was further investigated by incubating luciferase mRNA (1 μg) with air particulate extracts under conditions identical to those used for translation. Subsequent electrophoresis on 1% agarose (Fig. 4, lane 2) shows that RNA disappears completely following incubation with unfiltered extract. However,

TABLE 1. Toxicity of air particulates collected in residences with moisture problems

House no., room(s)	Fungus cultured	Toxin equivalents (ng/m³)[a]		Vol of air containing particulates causing 50% translation inhibition (m³)
		T-2	SG	
71, bedroom	*Stachybotrys* sp.	1.09	2.06	1.441
71, kitchen	*Stachybotrys* sp.	1.66	3.15	0.941
73, bedroom	*Stachybotrys* sp.	1.75	3.33	0.899
74A, bedroom	*Stachybotrys* sp.	0.98	1.86	1.600
74B, bedroom				
Test 1	NA[b]	0.50	0.94	3.152
Test 2		0.47	0.89	3.333
Test 3		0.54	1.02	2.900
75, baby's room	*Stachybotrys* sp.	1.10	2.09	1.421
75, toy room	*Stachybotrys* sp.	1.12	2.12	1.395
78, baby's room	*Rhizopus* sp.[c]	1.91	3.64	0.816
78, basement	*Rhizopus* sp.[c]	4.56	8.68	0.342
79A, bedroom	*Stachybotrys* sp.	17.90	34.10	0.087
79B, bedroom	*Stachybotrys* sp.	8.00	15.20	0.195
80, front room	*Stachybotrys* sp.	14.10	27.00	0.110
80, baby's room	*Stachybotrys* sp.	17.30	33.00	0.090
Control rooms (n = 3)	NA	0[d]	0[d]	NA
Control rooms (n = 2)	NA	0.01[d]	0.02[d]	NA
Control room	NA	0.09[d]	0.20[d]	NA

[a] r^2 values for fitted dose-response curves ranged from 0.950 to 0.998. T-2, T-2 toxin; SG, satratoxin G.
[b] NA, not available.
[c] Heavy growth precluded identification of other fungi.
[d] Single-dose measurement.

FIG. 4. Luciferase mRNA (1 μg) incubated with air particulate sample extracts. Lanes 1, control (water added instead of extract); 2, extract showing a high degree of luciferase translation inhibition; 3, the same extract as in lane 1 filtered through a Millipore Ultrafree-MC 5000 NMWL centrifuge filter; 4, 1 μg of standard luciferase mRNA.

incubation with the same extract filtered through Millipore Ultrafree-MC 5000 NMWL units did not lead to detectable degradation of RNA.

In order to quantitate the sensitivity of the translation assay to RNase, we used fungal RNase T_1 from *A. oryzae* (molecular mass, 11 kDa). As shown in Fig. 5, subpicogram amounts of RNase affect the translation of luciferase. Filtration through Millipore Ultrafree-MC 5000 NMWL fillers efficiently removes up to almost 2 ng of RNase. Increasing the concentration of RNase results in leaking of enzymatic activity through the filter. To be sure that the extracts were RNase free, they were filtered and assayed a second time by using the amount that reduced the translational activity by about 50% in the first assay. If the inhibitory effect on luciferase translation is reduced following the second filtration, it could be attributed to the presence of RNase in the extract. If observed changes remain within the limits of experimental error (several percent), we conclude that other inhibitors, most likely mycotoxins, were responsible for the inhibition of protein translation. To date, none of the environmental samples have shown reduced inhibition after the second filtration, demonstrating that the single filtration is sufficient to remove low levels of RNase present in the air particulate extracts.

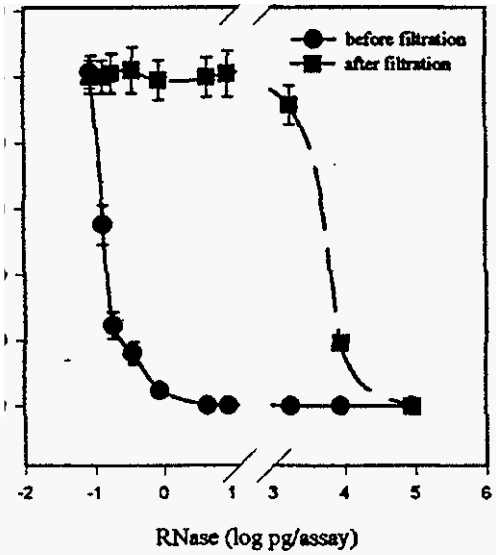

FIG. 5. Effect of T_1 RNase on luciferase translation. The inhibitory effect of RNase was measured before and after filtration through Millipore Ultrafree-MC 5000 NMWL units. The values are means ± SEM (n = 3).

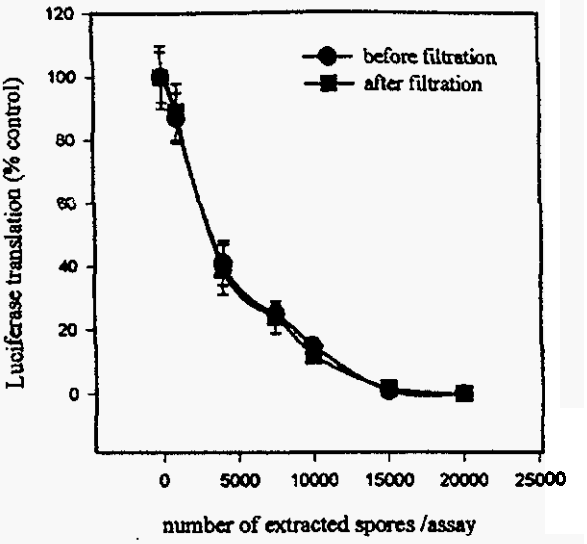

FIG. 6. Effect of *S. chartarum* spore extracts on luciferase translation in the rabbit reticulocyte system. Comparison of extracts filtered through Millipore Ultrafree-MC 5000 NMWL units with unfiltered extracts. The values are means ± SEM (n = 3).

Control experiments performed with pure T-2 toxin indicate that there is no loss of trichothecenes in extracts due to the filtration. T-2 toxin solutions can be passed through Millipore Ultrafree-MC 5000 NMWL units up to three times without any significant change in toxicity as estimated by the luciferase translation assay.

Because the focus of the tests is on fungal spores, it was important to determine if the spores contain detectable amounts of RNase activity. The presence of RNase in fungal spores has been reported (16, 30). *S. chartarum* spores were extracted in accordance with our standard ethanol procedure and used in the luciferase translation assay. The dose-response curves (Fig. 6) generated with extracts obtained before and after filtration through Millipore Ultrafree-MC 5000 NMWL units were virtually superimposable, indicating the lack of RNase activity in spore ethanol extracts. This suggests that either *S. chartarum* spores do *not* contain RNase activity or none of the enzyme is extracted or active under the conditions of the experiment.

In addition to removing traces of RNases, ultrafiltration would remove other possible interfering agents, such as proteases or endotoxin. We have not been able to detect any effect of endotoxin on luciferase translation at concentrations of up to 1 μg/ml.

Testing and quantitating the toxicity of environmental samples. The luciferase translation method was used to detect and quantitate the toxicity of air particulates collected in houses with known water and mold problems where the presence of toxigenic mold has been confirmed by culturing of bulk samples. Dose-response curves were generated by using filtered (Millipore Ultrafree-MC 5000 NMWL fillers) extracts of polycarbonate filters. Dose-response curves for T-2 toxin or satra-

toxin G were run in parallel with each experiment. The results are expressed as toxin equivalents per cubic meter of air determined by matching the 50% inhibition points of the experimental extract curves and the T-2 toxin and satratoxin G curves. The amounts of T-2 toxin and satratoxin G (nanograms) causing 50% inhibition were equated to the volume of extract (microliters) causing 50% inhibition of the luciferase translation. The volume of extract was then converted to the volume of air sampled (cubic meters), and toxin equivalents ("anagrams prr cubic meter) were obtained.

Table 1 shows the results of toxicity tests in several houses and rooms. The highest toxicity corresponds to about 17 ng of T-2 toxin or 34 "g of satratoxin G present in 1 m^3 of air. Either control rooms (clean rooms with no evidence of mold) had no detectable toxicity, or their toxicity was no higher than0.091 ng of T-2 toxin equivalents/m^3 (5 to 200 timer lower than that of contaminated rooms). Detecting toxicity of control rooms required using much larger amounts of extracts, corresponding to 5 to 10 m^3 of sampled air. With the routine sampling of residences yielding a maximum of 10 m^3 (8 hat 22 liters/min), only a single-point reading could be obtained

To further validate the testing procedure, the reproducibility of multiple screening was assessed. Table 1 (house 748) show results obtained after collecting air samples in the same room three times for 8 h each time within a period of 72 h. The three separate samplings yielded toxin equivalent values of 0.502, 0.471, and 0.54 (mean, 0.505; standard error of the mean [SEM], 0.02) ng/m^3.

DISCUSSION

The aim was to develop a rapid and inexpensive method to quantitatively assess exposure to trichothecenes as a biomarker for toxigenic fungi such as *S. chartarum*, which has recently been linked to an outbreak of pulmonary hemosiderosis in infants (see reference 10). Currently, airborne exposure to toxigenic fungi can only be estimated based on the results of culturing or spore counting of air particulate samples. Airborne concentrations of detected culturable spores are often falsely low (1, II). Furthermore, because different isolates of the same fungal species can produce various amounts of mycotoxins, depending on the growth conditions (18, 21), the isolation of a toxigenic fungus from a building cannot be take" as a" indication of the level of toxin exposure. Spores that have lost the ability to germinate still contain stable trichotheccnc mycotoxins. Toxicity tests may confirm both the presence and the toxic potential of fungal isolates in a particular home environment. Thus measurement of total trichothecene toxicity rather than rhc number of viable sporcs is a morc accurate approach.

Existing literature on cytotoxic effects of fungal spores collected on polycarbonate filters and pure mycotoxins suggests that cytotoxicity assays may be suitable for evaluation of inhalation exposure to toxigenic fungi (12, 14). Cytotoxicity has been used to measure toxic effects of fungal spores under controlled experimental conditions. Pasanen and coworkers (22) employed the fetal lung cell-based assay to demonstrate toxicity of airborne spores of S. *chartarum* growing in the laboratory on substrates such as hay. grain, and wallpaper. that have been sterilized prior to fungal contamination. in a study of problem buildings using kidney cells *and an* MTT *test,* Gareis (12) demonstrated the cytotoxicity of fungus-contaminated samples of gypsum board. However, the specificity and quantitative aspect of those assays have not been tested in large practical building surveillance studies.

Cytotoxicity experiments employing the MTT assay described in this report demonstrate that porcine kidney (PK15) cells are highly susceptible to pure trichothecene toxins. Similar midpoint toxicity values for T-2 toxin of 2.8, 5.6. and 9.8 ng/ml for melanoma cells, keratinocytes, and hepatoma cells were reported by others using the neutral red assay (2). The MTT assay used with MDBK cells yielded 50% inhibition at I to 1.5 ng of T-2 toxin per ml (15). Cytotoxity experiments performed with numerous mycoroxinr and a different line of swine kidney cells yielded 0.8 μg of DON per ml and 6.2 μg of satratoxin G per ml for 80% MTT cleavage activity (14).

By using extracts of polycarbonate filters which have been exposed to the air in houses with mold and moisture problems, we were unable to detect any cytotoxic effect on PK15 cells. In contrast, bulk samples collected in parallel with the air samples exhibited very high cytotoxicity. This was expected, since bulk samples. in general, contain much higher concentrations of microbes than can be found in air particulates (II). It seems that at least some of that toxicity could be attributed to the presence of bacterial endotoxin and other high-molecular-weight compounds. In most cases, filtration of ethanol extracts through Ultrasart D20 filters, which removes molecules larger than 20 kDa, led to reduction of the observed cytotoxicity. In summary, we find the MTT cleavage-based cytotoxicity assay to be suitable for screening of highly toxic bulk samples. especially after removal of endotoxin and other high-molecular-weight compounds. but not sufficiently sensitive or specific to detect and quantify the trichothecene toxicity of air particulates. Hi-flow pumps with impingers collecting much larger samples may help solve the sensitivity problem. but this does not appear to be practical for routine sampling. especially in residential buildings. The lack of specificity toward fungal toxins and the potential for synergistic effects do not appear to be readily resolvable in this system.

The primary mode of trichotheccnc action in living cells is inhibition of the protein translation process (6). Assays based on protein synthesis have been used to detect and compare mycotoxins, as well as to study their mechanism of action (27, 28). Translation inhibition-based tests performed with cultured cells appear to be highly sensitive to trichothecenes, with T-2 toxin 50% inhibition values of 10 to 15 ng/ml for CHO cells and 1 ng/ml for MDBK cells (15). T-2 toxin has been shown to be much less effective in cell-free translation systems, such as rabbit reticulocyte lysates requiring microgram-per-milliliter concentrations (27). The protein translation assays previously described all involve the use of radioactive amino acids and require several hours to several days to complete the tedious and labor-intensive processing of samples.

In recent years, the translation of firefly luciferase has been used in molecular biology as a nonradioactive alternative to detect and quantitate the expression of reporter genes and as a control for in vitro translation. The luminescence of in vitro-translated luciferase can be easily detected and quantified. Luciferase catalyzes ATP-dependent conversion of luciferin to oxyluciferin with concomitant release of light The quantum yield of this reaction is the highest in efficiency of my known biological reaction (26). The light emitted from firefly luciferase is directly proportional to tbc number of luciferase enzyme molecules whc" the substrate is not in excess (7). Luciferase activity can be measured directly in the translation mixture within seconds. The entire testing procedure. including pwtci" translation. can be completed in less than 2 h. The rabbit reticulocyte system has been extensively studied and optimized to yield functional, biologically active proteins (13) and is currently available from several commercial sources.

Wc have demonstrated that the trichothecenes T-2 toxin, satratoxin G. and DON readily inhibit the translation of firefly

TABLE 2. Comparison of trichothecene detection limits obtained by different methods

Toxin and parameter	Luciferase translation assay[a]	MTT cytotoxicity assay[b]	MTT/LT[b]	Cytotoxicity[c,d]	Thin-layer chromatography[e]	Enzyme-linked immunosorbent assay[e,f]
T-2 toxin						
Concn at 80% activity (pg/ml)	20,000	1,000				
Vol used (μl)	0.25 (1)	100				
Amt detected (pg)	5 (20)	100		10–40,000	500,000	1–40
Ratio			5–20			
Satratoxin G						
Concn at 80% activity (pg/ml)	50,000	10,000				
Vol used (μl)	0.25 (1)	100				
Amt detected (pg)	12.5 (50)	1,000		630,000		
Ratio			20–80			
DON						
Concn at 80% activity	200,000	200,000				
Vol used (μl)	0.25 (1)	100				
Amt detected (pg)	50 (200)	20,000		100,000	500,000	250,000
Ratio			100–400			

[a] The value in parentheses is the practical volume that can be accurately dispensed. This volume is four times larger than the amount needed for the luciferase assay and results in an increase in the detection limit (see text).
[b] This report. LT, luciferase translation assay.
[c] Robb and Norval (25).
[d] Hanelt et al. (14).
[e] Dietrich et al. (8).
[f] Casale et al. (5).

luciferase mRNA in a cell-free rabbit reticulocyte system. T-2 toxin and, to a lesser extent, satratoxin *G* are not as effective in the reticulocyte lysate as they are in PK15 cells. The greater potency in intact cells can k explained by toxicity independent of translational mechanisms such as effect on membranes or additional toxicity of toxin metabolites (4, 29). In the case of DON, only a small difference in potency between PK15 cells. and the luciferase translationsystem was dctcctcd. DON contains different specific side groups than T-2 toxin and inhibits both the elongation and termination steps of the protein translation process, whereas T-2 toxin inhibits the initiation step (9, 27).

Although the effective toxin concentrations in the luciferase translation assays may bc similar to (as inthe casc of DON) or even higher than (as in the cases of T-2 toxin and Satratoxin *G*) those in the cytotoxicity assay. the practical sensitivity advantage of the luciferase translation assay results from the very small volume of extract that can be used. This is evident from Table 2, which contains a comparison of the detection limits of the luciferase translation method and the MTT cytotoxicity assay described in this report, as well as methods described by others including cytotoxicity tests, thin-layer chromatographic analysis, and immunodetection. The luciferase translation ten is a two-step procedure composed of a protein vanslation step and a luciferase assay step. Only 0.25 μl of vanslation mixture is required to obtain readings of about 60,000 RLU. Practically, one is not able to attain this limit in the first step by using regular pipetting, by which only I μl of translation mixture can be accurately dispensed. This increases the practical dttcction limit by a factor of four. hence, the difference between the real and the practicsl (in parentheses) volumes and amounts detected (Table 2). *Use of robotic devices* allowing accurate dispensing of nanoliter volumes should close the gap between the reel and practical detection limits. The sensitivity of the assay remains comparable to the range of immunodetection. However. unlike immunodetection, the luciferase translation assay does not require an array of specific antibodies and measures combined toxicity rather than concentrations of individual toxins, thus providing a broader assessment of exposure. Toxicity analysis of problem houses finds toxin levels corresponding to nanogam amounts of T-2 toxin and satratoxin *G*. which is almost 1,000 times more than our practical detection limits. Furthermore, the procedure is conveniently standdrdircd by parallel determination of T-2 toxin and satratoxin *G* dose-response curves. Although our use of rabbit reticulocytes yields very reproducible results, some batches of lysate occasionally demonstrate slightly different initial activity and altered sensitivity to trichothecenes, especially after prolonged storage. Therefore. using standard toxins provides an additional measure of interexperiment reproducibility and allow the expression of toxicity in terms of T-2 toxin and/or satratoxin G equivalents. The choice of standard toxins is somewhat arbitrary. Initially, T-2 toxin was selected because of its commercial availability and low cost and the relative abundance of availabk literature. Satratoxin *G* was subsequently included because it was detected in isolates of *S. chartarum* from water-damaged houses in Cleveland, Ohio, included in a study of pulmonary hemosiderosis in infants (18). However, this toxin is not commercially available. and the literature describing its action is rather scarce (17). Although possible effects of RNases and proteases. as well as other. unidentified substances interfering with protcin translation, are potentially serious limitations of the assay, we have largely excluded these problems by the double filtration of extracts to remove enzymes and other large molecules. Exclusion of RNases and certain biomarkers, such as endotoxin, indicative of the presence of gram-negative bacteria allow one to focus on trichothecene mycotoxins as an indicdtor of exposure to toxigenic *fungi*.

In addition to its sensitivity, the luciferase translation assay yields highly reproducible results, as demonstrdted by multiple screening of the same moisture problem house. As an activity assay, the luciferase translation test does not provide the toxin composition of environmental samples, which can only be in-

vestigated by using costly multimethod approaches, as described by Andersson et al. (1). Rather, the luciferase translation assay is a practical, rapid, and inexpensive means to detect and quantify the fungus-derived toxicity of air particulates from problem indoor environments. Inclusion of this assay in a battery of fungal tests to assess indoor environments is planned in order to demonstrate its projected utility.

ACKNOWLEDGMENT

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